EFFECTS OF UNIALGAL AND BACTERIA-FREE CULTURES OF *Gymnodinium brevis* ON FISH

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THE EFFECTS OF UNIALGAL AND BACTERIA-FREE CULTURES OF GYMNODINIUM BREVIS ON FISH AND NOTES ON RELATED STUDIES WITH BACTERIA

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ABSTRACT

This investigation was conducted to determine the effects of Gymnodinium brevis Davis, a naked dinoflagellate definitely associated with the sporadic mass mortality of marine animals in the Gulf of Mexico, on fish under controlled conditions. A series of experiments, seven with unialgal cultures and two with bacteria-free cultures, demonstrated the toxicity of this organism to six species of fish. Bacteria-free cultures were just as toxic as unialgal cultures. The cultures employed contained 0.6 to 4.8 million G. brevis per liter. Apparently the test fish were differentially sensitive to G. brevis cultures. In order of decreasing sensitivity, the test fish were: Membras vagrans, Mugil cephalus, Fundulus grandis, Mollienisia latipinna, Fundulus similis, and Cyprinodon variegatus. The lethality of bacteria-free G. brevis cultures to fish clearly indicates that this dinoflagellate is the direct cause of the mass fish mortalities with which its "blooms" are associated.

Toxicity of G. brevis cultures does not depend on the presence of living organisms. The more toxic portion of the cultures passes through a millipore membrane whereas it is retained by filter paper. In some experiments such possible lethal factors as oxygen deficiency, suffocation due to clogging of gills with masses of organisms, and bacterial growth were eliminated thereby establishing that G. brevis produces a toxic substance(s).

Two chromogenic marine bacteria, Flavobacterium piscicida Bein and an unidentified red-pigment-producing form, from the west coast of Florida were tested for toxicity to fish. The results of these tests are discussed.

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infested waters which contained numerous other organisms. In addition, raw samples were probably held under conditions unsuitable for the survival of G. brevis.

The next approach to this problem was to obtain bacteria-free or pure cultures of G. brevis. This isolation is necessary to determine whether a cause and effect relationship exists between G. brevis and the catastrophic fish kills. Furthermore, studies of such problems as the nutritional requirements of G. brevis, nature of the toxic substance, role of associated organisms, and effects of physical and chemical factors may be facilitated with the use of bacteria-free cultures since the uncertainty regarding the effects of associated bacteria would be eliminated.

The laboratory studies mentioned above, in coordination with field studies, will provide a better understanding of why these mass mortalities occur. Such knowledge will be helpful in predicting when and where outbreaks may be expected and in determining the feasibility of control measures.

We report[2/] herein the results of studies on the effects of unialgal and bacteria-free cultures of G. brevis on fish as well as the effects of some bacteria isolated from unialgal cultures of this organism and from waters off the west coast of southern Florida. Based upon the results of studies with bacteria-free cultures, we conclude that G. brevis produces the toxic substance(s) responsible for the mass mortality of marine animals associated with "blooms" of this organism in the Gulf of Mexico.

PROCEDURES FOR TESTING STERILITY AND ENUMERATING ORGANISMS

Bacteria-free cultures were grown in the same medium prescribed by Wilson and Collier (1955) and have been carried through several subcultures. After 10 months they have shown no apparent diminution in vigor. Several media were used to establish sterility. All G. brevis cultures originated from a culture obtained from a sample collected in a "bloom" which occurred near the coast of Florida in September, 1953. The details of procedures for culturing G. brevis, and methods used for obtaining bacteria-free cultures will be presented in another paper.

Sterilty Testing Media

Cultures of G. brevis used for transferring were tested for sterility in media prepared according to Spencer (1952): (1) peptone sea water (0.5%, bacto-peptone, 0.01%, $FePO_4$ dissolved in

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2/ We are indebted to Mr. K. T. Marvin, Mrs. Alice Kitchel, and Miss Jean Gates for assistance in performing the experiments reported herein and Messrs. E. A. Arnold and R. S. Wheeler for identifying the test fish.

75% aged sea water) and (2) peptone sea water agar (peptone sea water plus 1.5% bacto-agar). These media as well as all other sterility testing media subsequently described were dispensed in screw-cap tubes and autoclaved at 121° C. for 15 minutes.

We frequently used four other media similar to those employed by Droop (1954) for routine sterility testing. These media included: (1) distilled water liquid, (2) distilled water agar, (3) sea water liquid, and (4) sea water agar. Our media contained the following substances: 0.5% dextrose, 0.1% Difco neopeptone, 0.4% bacto-beef extract, 0.5% bacto-yeast extract, 0.015% sodium acetate ($NaC_2H_3O_2.3H_2O$), and soil extract (2.0 ml/100 ml). These substances (dextrose was often excluded) with and without bacto-agar (1.5%) were dissolved in both distilled water and 75% aged sea water to give the four combinations mentioned above. Droop (1954) listed the substances but not the quantities used. A personal communication (1956), however, revealed that his formula contained the organic substances in concentrations which were roughly 10 to 15 times less than the quantities given above. Furthermore, he included bacto-tryptone, which was not listed in his paper whereas we used Difco neopeptone. Subsequent to the completion of the present studies, the absence of bacteria from several G. brevis cultures was confirmed with media of Droop's formulation and also with these media diluted to 10 percent.

Other media used to supplement the routine tests included: (1) the sterility-test medium used by Sweeney (1954) containing 0.05% bacto-peptone, 0.0136% sodium acetate ($NaC_2H_3O_2.3H_2O$), 0.0202% KNO_3, 0.00356% K_2HPO_4, 0.00016% $FeCl_3.6H_2O$, and 0.000012% $MnCl_2.6H_2O$ dissolved in 75% aged sea water with and without bacto-agar (1.5%); (2) Spencer's peptone sea water media supplemented with 0.1% bacto-yeast extract as employed in medium 2116E (Morita and ZoBell, 1955); (3) semisolid medium composed of 0.075% trypti-case (Baltimore Biological Laboratory), 0.075% bacto-peptone, 0.075% bacto-yeast extract, 0.01% sodium acetate ($NaC_2H_3O_2.3H_2O$), and 0.2% Difco special (Noble) agar dissolved in aged sea water; (4) one percent bacto-peptone in aged sea water with and without bacto-agar (1.5%), the medium used by Bein (1954) to isolate and cultivate certain chromogenic bacteria found in Florida waters; and (5) Spencer's (1952) casein sea water agar composed of 0.05% bacto-peptone, 0.05% bacto-isoelectric casein, 0.05% soluble starch, 0.1% (v/v) glycerol, 0.02% K_2HPO_4, and 1.5% bacto-agar dissolved in 75% sea water.

Sterility tests for anaerobic bacteria were conducted occasionally with three different media: (1) bacto-fluid thioglycollate medium rehydrated with both distilled water and 75% aged sea water; (2) the general anaerobic medium (slightly modified) used for marine bacteria by Morita and ZoBell (1955) containing 0.5% bacto-peptone,

0.1% bacto-yeast extract, 0.01% $FePO_4$, 0.1% sodium formaldehyde-sulphoxylate, and 0.0001% resazurin dissolved in 75% aged sea water with and without bacto-agar (1.5%); and (3) an anaerobic medium prepared by adding 0.01% sodium thioglycollate to the semisolid medium described in the previous paragraph. The melted general anaerobic agar medium was cooled to 40-42° C. prior to adding the test culture which was mixed by swirling the tube before the agar solidified. After adding the test culture, sterile melted "vaspar" (50% vaseline and 50% paraffin) was poured into each tube of anaerobic medium, except fluid thioglycollate medium, to exclude oxygen.

Inoculation and Incubation Procedures

All sterility tests, unless otherwise indicated, were made with 1.0 ml of test culture in 10.0 ml of medium. The agar media were inoculated in the following ways: (1) pour-plate---mixing test culture in a sterile Petri dish with melted medium cooled to 40-42° C., (2) streak-plate---streaking 0.1 ml of test culture on a freshly prepared plate, (3) stab culture---placing 0.1 ml of test culture into medium in screw-cap tubes (20 mm x 125 mm) and then stabbing an inoculating needle to the bottom, and (4) slant cultures---placing the test culture on freshly slanted medium in screw-cap tubes. Slant cultures were generally prepared for most routine tests. The agar plates were sealed with masking tape to prevent desication and mold contamination while incubating. Semisolid medium was inoculated by stabbing to the bottom with a micro-pipette and then gradually releasing the inoculum as the pipette was slowly withdrawn.

We incubated the sterility-test cultures in the dark at 28-30° C. for a minimum of 6 weeks before discarding them as sterile. This temperature level was selected since some of the bacteria isolated from the unialgal cultures of G. brevis appear to grow more slowly at 24-25° C.

On one occasion the sterility of several cultures was tested in duplicate in various liquid and agar media; the four methods for inoculating agar cultures were used. One set was incubated with illumination (175-300 ft-ca.) and temperature (24-25° C) the same as used for G. brevis cultures; the other set was incubated in the dark at 28-30° C. After 6 weeks none of the cultures showed either visible colonies or cloudiness of any sort except an occasional mold or bacterial colony on the surface of a few streak- and pour-plates.

We attribute the occasional appearance of mold or bacterial colonies in our test cultures, especially on the surface at the periphery of streak- and pour-plates, to contamination while the

plates were exposed by necessary manipulations. The position of the colonies as well as the appearance of similar colonies on some control plates (uninoculated agar plates), which were treated in the same manner as the test cultures, supports this conclusion. We rarely encountered accidental contamination of sterility-test culture contained in screw-cap tubes.

Miscellaneous Checks for Sterility

We consider that the medium used for culturing G. brevis is unlikely to be suitable for the growth of photosynthetic bacteria. Nevertheless, a few cultures were checked for such organisms. The checks were made with a medium developed by Dr. T. J. Starr of this laboratory for the isolation of marine non-sulfur purple bacteria. This medium is composed of: 0.2% sodium acetate ($NaC_2H_3O_2.3H_2O$), 0.05% $Na_2SO_3.7H_2O$, 0.01% $MgSO_4.7H_2O$, 0.05% K_2HPO_4, 0.1% $(NH_4)_2SO_4$, 0.0001% $FeCl_3.6H_2O$, 2.5% NaCl, 0.01% bacto-yeast extract, 0.01% sodium thioglycollate, and 1.5% Difco special (Noble) agar dissolved in double distilled water. Just before inoculation the medium was melted, and sterile $NaHCO_3$ solution was added aseptically to each tube to give a 0.1% concentration and a final pH of 8.0. This medium was inoculated and treated in the same manner as previously described for the general anaerobic medium. These sterility-test cultures, which were incubated under the same light and temperature conditions used for G. brevis, showed no evidence of growth after 6 weeks.

Phase-contrast microscopic examination (970X) of wet preparations of a few G. brevis cultures which were determined to be pure by cultural methods did not reveal any contaminating organisms. These examinations, conducted several months after the initial establishment of bacteria-free cultures, were performed to check for possible contaminants which may have maintained themselves in G. brevis culture medium after repeated subculturing, and yet not have grown in any of the sterility-test media employed.

Procedure for Enumerating Bacteria

Aerobic bacterial counts presented in the experiments to follow were estimated by plating serial dilutions of the test sample. The dilution water blanks (75% aged sea water) dispensed in 9-ml amounts in screw-cap tubes were autoclaved at 121° C. for 15 minutes. Serial dilutions of 1:10; 1:100; 1:1,000; 1:10,000; and 1:100,000 were prepared of each sample to be counted. One ml of each dilution and 10.0 ml of melted Spencer agar, cooled to 40-42° C., were mixed in a sterile Petri dish by gentle swirling before the agar hardened. Because of the possibility of low counts a plate was also prepared with 1.0 ml of undiluted sample. After the agar hardened, the plates were sealed with masking tape and incubated in an inverted position for 4 to 7 days at 28-30° C. Colonies were enumerated

with the aid of a Quebec colony counter. Plates with either more than 300 or less than 30 colonies were not used in quantitative estimates except in a very few instances. The exceptions were in cases where either the most dilute plates contained more than 300 colonies or the undiluted plate contained less than 30 colonies.

The bacterial counts most likely represent minimal concentrations because nutritional and environmental requirements of an entire bacterial population cannot be satisfied with any one medium or with a single set of incubation conditions. We made no attempt to enumerate anaerobic bacteria. All colonies except those with the typical appearance of molds were counted, therefore, any microorganisms producing bacteria-like colonies were included in the counts.

Since we could not prepare pour-plates of all water samples immediately after collecting them, another possible source of error in the counts should be considered. Both quantitative and qualitative changes in the bacterial population may have occurred before some of the samples were plated, particularly those plated several hours or even days after collection. Immediately after collection all water samples were refrigerated (4° C.) until shortly before preparing the plates. In most cases the storage period did not exceed 6 hours. However, this period varied considerably in some experiments, especially those in which several samples were counted. Consequently, we have recorded the extremes of the storage period for each experiment.

Procedure for Enumerating Dinoflagellates

The concentration of G. brevis and other dinoflagellates was determined in two steps: (1) a preliminary counting of 1.0 ml, 0.1 ml, and 0.01 ml aliquots from a sample mixed by gently swirling the tube (vigorously shaking frequently causes many of the organisms to cytolyse) to determine sample size best for counting, and (2) counting 3 to 9 aliquots of the quantity selected in the first step. The latter counts were averaged to obtain the G. brevis concentrations. The counts probably represent minimal levels because the organism tends to disintegrate when manipulated. Because of this tendency, only one aliquot was withdrawn at a time and it was counted immediately. A wide field stereoscopic microscope with a magnification of 54X was used in making the counts.

EXPERIMENTS WITH UNIALGAL CULTURES OF GYMNODINIUM BREVIS AND OTHER DINOFLAGELLATES

Seven experiments testing the toxicity to fish of unialgal cultures of G. brevis and some other dinoflagellates were performed. All of these studies, even those which are preliminary such as experiments 1 through 3, are presented because the details and results vary considerably in some cases. In some experiments only one test fish

was used per container because either the available fish were too few or the containers were too small to accommodate more. Moreover, duplicate containers were not always used because of limitations imposed by insufficient supply of either test fish or test materials. We have taken special care to record all experimental details, some of which may be of no significance, since they may prove of value to others in reviewing our work.

Experiment 1. A Simple Test of the Toxicity of *Gymnodinium brevis* Cultures

This experiment was conducted to determine whether unialgal *G. brevis* cultures would kill fish. We used a $3\frac{1}{2}$-week-old culture, replenished with fresh medium three times weekly, containing 1.8 million organisms per liter. Sea water from a lagoon (at the east end of Galveston Island, Texas), the locality where the test fish were collected, served as control material. The test materials were not aerated. One rough silverside (*Membras vagrans*) $3\frac{1}{2}$ in. long and one sailfin molly (*Mollienisia latipinna*) $2\frac{1}{2}$ to 3 in. long was placed in each of two 1-liter beakers--one containing sea water, the other *G. brevis* culture. The beakers were covered with polyethylene sheeting.

The *M. vagrans* survived only 4 minutes in the *G. brevis* culture whereas this species survived 43 minutes in the sea water. The *M. latipinna* died after 85 minutes exposure to the *G. brevis* culture and the fish in the sea water was alive when the experiment was discontinued 4 days later. Although the lethality of *G. brevis* cultures to fish is evident from these results, they do not necessarily prove that a toxic substance is involved.

Experiments 2 and 3. Comparison of Effects of *Gymnodinium brevis* and *Gymnodinium splendens* Cultures

The mere presence of numerous dinoflagellates may have been responsible for the toxicity of the unialgal culture used in experiment 1. To test this possibility, fish were subjected to unialgal *G. brevis* and *G. splendens* cultures in experiments 2 and 3. Experiment 2 was conducted under the same conditions as experiment 1. A 4-week-old unialgal culture of *G. brevis* and a 10-week-old unialgal culture of *G. splendens* containing 2.1 and 2.8 million organisms per liter, respectively, were tested for toxicity to *M. latipinna* ($2\frac{1}{2}$ to 3 in. long). Both cultures were replenished with fresh medium three times weekly during the incubation period. Sea water from which the test fish were taken was used as control. One fish was placed in each of three test materials. The fish in the *G. brevis* culture died after 47 minutes. In the *G. splendens* culture and in sea water the fish were alive at the close of the study 19 days later.

Experiment 3 duplicates experiment 2 in most respects except that the cultures were a week older. At this time there were 2.0

million G. brevis and 2.6 million G. splendens per liter in the cultures. The M. latipinna ($2\frac{1}{2}$ to 3 in. long) lived only 68 minutes in the G. brevis culture, but they were alive in the G. splendens culture and sea water 3 days later when the experiment was discontinued.

The excellent survival of the fish in G. splendens cultures in contrast to the lethality of G.brevis cultures indicates that the latter cultures contained a toxic substance(s). Since the cultures of G. brevis were not pure, the toxic substance could have been produced by G. brevis, the associated bacteria, or both.

Experiment 4. Effects of Unialgal Gymnodinium brevis Cultures and the Associated Bacterial Flora

A series of experiments (4, 5, 6, and 7) was designed mainly to determine whether G. brevis or its associated bacterial flora is responsible for the toxic effects of unialgal cultures to fish. If the bacteria prove non-toxic under the same cultural conditions, one could reasonably assume that G. brevis produces the toxic substance. Much of the value with regard to the original purpose for conducting these experiments has been lost subsequent to the development of mass bacteria-free cultures of G. brevis. Bacteria-free cultures made it possible to demonstrate experimentally that G. brevis produces a fish-killing substance. The details are presented later in this paper.

To obtain some of the test materials used in these experiments (4, 5, 6, and 7), 20 liters of culture medium were prepared, 5 liters of which were placed in each of two Pyrex bottles ($2\frac{1}{2}$-gallon); another Pyrex bottle (5-gallon) received the remaining 10 liters. Each bottle of medium was heated to 75° C.(5 to 6 hours heating required) on three successive days to reduce the bacterial load. One of the $2\frac{1}{2}$-gallon bottles (No. 1) was inoculated with 10.0 ml of a 6-week-old unialgal G. brevis culture with a bacterial count of 8.1 million per ml. The other $2\frac{1}{2}$-gallon bottle (No. 2) was seeded with 10.0 ml of G. brevis-free inoculum in an attempt to culture the associated bacteria. This inoculum, having a bacterial count of 10.3 million per ml, was obtained by heating between 37-39° C. for 30 minutes a portion of the same culture used to inoculate bottle 1. The 5-gallon bottle (No. 3) containing uninoculated medium was arranged so that bottles 1 and 2 could be replenished from this reservoir when culture materials were removed for toxicity tests and chemical analyses.

Samples were taken from the three bottles at irregular intervals during the first 25 days of incubation to follow the bacterial growth. The bacterial counts (Table 1) of samples taken at 1-, 4-, 14-, and 25-day intervals from the unialgal G. brevis culture (bottle 1) and the G. brevis-free bacterial culture (bottle 2) were comparable except for the 4-day samples. The 4-day sample from the G. brevis

Table 1.--Bacterial counts, at irregular intervals, of unialgal G. brevis culture, companion G. brevis-free culture, and replenishment medium from common reservoir

Bottle No.	Incubation period (days)	No. bacteria per ml (millions)	Remarks
1 (Unialgal G. brevis)	Shortly before inoculation	0.000070	Media cooled to room temperature after final heating before collecting sample; plates prepared shortly thereafter.
2 (G. brevis-free bacteria)		0.000090	
3 (Reservoir, uninoculated)		---	
1	Shortly after inoculation	0.014	Plates prepared shortly after collecting samples.
2		0.018	
3		---	
1	1	0.37	Samples refrigerated 5 days before preparing plates.
2		0.41	
3		0.0015	
1	4	2.7	Samples refrigerated 2 days before preparing plates.
2		1.8	
3		0.26	
1	14	2.0	Plates prepared 15 to 20 minutes after collecting samples.
2		1.8	
3		0.13	
1	25	1.4	Samples refrigerated 2 days before preparing plates.
2		1.2	
3 1/		12.2	

1/ The value of 12 million bacteria per ml for the reservoir appears to be excessively high when compared with the other counts obtained at either earlier or later intervals. With the exception of the presently considered value, the highest bacterial count obtained from the reservoir was 1.8 million per ml (Table 5, Experiment 7).

culture had a bacterial count of 2.7 million per ml--about 50% greater than that of the G. brevis-free bacterial culture. The validity of some bacterial counts is questionable because of prolonged refrigeration of samples before preparing the plates. They indicate, however, the relative number of bacteria in the three bottles at the various sampling intervals. Although bottles 1 and 2 apparently were inoculated with the same bacterial flora, we can only presume that the floras subsequently developing in these bottles were qualitatively comparable.

Approximately 6 weeks after inoculating bottles 1 and 2, materials from these bottles and the reservoir (bottle 3) in addition to an 11-month-old unialgal G. brevis culture and centrifuged sea water were used to conduct experiment 4. One striped mullet (Mugil cephalus) $2\frac{1}{2}$ to 3 inches long was subjected to each of the five different nonaerated test materials, 750 ml in 1-liter beakers. We observed the fish closely and recorded the time at which they began to show imbalance ("distress time") and the time at which they showed no visible opercular movement ("death time"). Bacterial-count samples were obtained from each container before the fish was added. These samples were refrigerated 1 to $1\frac{1}{2}$ hours before plating.

The results (Table 2) of this 24-hour experiment show that the fish in the two unialgal G. brevis cultures died in 50 minutes and $2\frac{1}{4}$ hours. Two of the three control fish survived considerably longer, $7\frac{1}{2}$ hours in the G. brevis-free bacterial culture and the entire test period in the uninoculated culture medium. However, the fish in the centrifuged sea water died after 58 minutes. The early death of this control fish was perhaps due to injury.

The bacterial count of 6.0 million per ml for the G. brevis culture (bottle 1) in container 3 was five times greater than that for the G. brevis-free bacterial culture (bottle 2) in container 4. Prior to this 6-week check the bacterial counts of these two cultures were comparable (Table 1). The disparity in the bacterial counts for experiment 4 necessitated additional studies in order to determine the toxicity agent in unialgal G. brevis cultures.

Experiment 5. The Effects of Unialgal Gymnodinium brevis Cultures, the Associated Bacterial Flora, and Unialgal Prorocentrum sp. Cultures

In addition to testing the effects of G. brevis culture (bottle 1) and G. brevis-free bacterial culture (bottle 2), another dinoflagellate, prorocentrum sp., was tested for toxicity to fish in the second experiment of this series. This organism was isolated from the lagoon, Galveston, Texas. The materials in bottles 1 and 2 were $3\frac{1}{2}$ months old at this time. Freshly collected sea water served as control. The four different materials, 2 liters of each in 4-liter beakers,

Table 2.--Effects of unialgal G. brevis cultures and a G. brevis-free culture presumed to contain the bacterial flora associated with this organism in unialgal cultures on Mugil cephalus--experiment 4

Container No.	Material in container	Distress time[1] (hr:min)	Death time[2] (hr:min)	No. bacteria per ml (millions)	Remarks
1	Centrifuged sea water (Galveston beach)	0:50	0:58	0.0070	--
2	11-month-old G. brevis culture, 0.8 million organisms per liter	0:05	0:50	10.2	Fresh medium added to G. brevis culture occasionally
3	6-week-old G. brevis culture (bottle 1), 0.6 million organisms per liter	0:15	2:15	6.0	Fresh medium added to G. brevis culture occasionally
4	6-week-old G. brevis-free bacterial culture (bottle 2)	none[3]	none	1.2	Fresh medium added to bacterial culture occasionally
5	6-week-old medium from reservoir (bottle 3)	not known	7:35	0.080	--

1/ Time required for fish to show first signs of imbalance.
2/ Time of cessation of opercular movement.
3/ None indicates that distress or death did not occur during the 24-hour test period.

were tested in duplicate for toxicity to Mugil cephalus ($2\frac{1}{2}$ in. long). One fish was tested in each container without aeration. Samples were collected from each container for bacterial counts before the fish was added. These samples were plated after $1\frac{1}{2}$ to 5 hours refrigeration.

The test fish subjected to the G. brevis culture died within an hour (29 and 47 minutes) whereas the fish in the other test materials lived a minimum of 8-1/3 hours to a maximum of 24 hours--the duration of the experiment (Table 3). The bacterial counts of both the G. brevis culture (bottle 1) and the G. brevis-free bacterial culture (bottle 2) had decreased since experiment 4 was conducted. Just as in experiment 4, however, the G. brevis culture had a much higher count--2.9 to 3.4 million bacteria per ml in contrast to 0.20 to 0.23 million per ml for the G. brevis-free culture.

One of the containers of G. brevis culture (6) used in experiment 5 was employed in a supplementary study to determine whether adding several fish to the same culture would affect its toxicity. Another phase of this study was to check the response of fish transferred to sea water after being subjected to G. brevis culture. Immediately after the fish in container 6 died (29 minutes after beginning experiment 5) it was removed and the first of five additional M. cephalus were placed in this container. This fish succumbed after 21 minutes exposure. After removing the dead fish, the second one was allowed to remain in container 6 for 15 minutes. It was then transferred to sea water (container 1) where it died 12 minutes later. Fifteen-minute and 7-minute exposures, respectively, in container 6, were required to kill the third and fourth fish. Each fish was removed from the container after it died. After 3 minutes exposure in container 6, the fifth one was removed to container 1 where it survived for 2-3/4 hours.

Experiments 4 and 5 (Tables 2 and 3) were inadequately controlled with regard to quantities of bacteria. Experiment 6 was performed in an attempt to correct this shortcoming.

Experiment 6. Comparison of Toxicity of Unialgal Cultures of Gymnodinium brevis and Prorocentrum sp., and Effects of Heating and Filtration on Toxicity

Besides attempting to ascertain the source of the toxic substance in unialgal G. brevis cultures, this experiment included a study of the effects of heating and filtration on the toxicity of such cultures.

One month prior to conducting experiment 6, the remaining portion of the G. brevis-free bacterial culture (bottle 2) received an inoculum of unialgal Prorocentrum sp., which had proved nontoxic to M. cephalus in experiment 5 (Table 3). This step was taken in an attempt to increase the bacterial concentration in bottle 2 to a level comparable to that in the G. brevis culture (bottle 1). Centrifuged sea water

Table 3.--Effects of unialgal cultures of G. brevis and Prorocentrum sp. and a G. brevis-free culture presumed to contain the bacterial flora associated with this organism in unialgal cultures on Mugil cephalus--experiment 5

Container No.	Material in container	Death time[1] (hr:min)	No. bacteria per ml (millions)	Remarks
1	Freshly collected sea water (Galveston beach)	Between 10 and 22 hours	0.0025	--
2	Same as container 1	Between 10 and 22 hours	0.0030	--
3	3-month-old Prorocentrum sp. culture, 0.5 million organisms per liter	none[2]	0.59	Fresh medium added to Prorocentrum culture occasionally
4	Same as container 3	Between 10 and 22 hours	0.46	--
5	3½-month-old G. brevis culture (bottle 1), 1.9 million organisms per liter	0:47	2.9	Fresh medium added to G. brevis culture occasionally
6	Same as container 5	0:29	3.4	--
7	3½-month-old G. brevis-free bacterial culture (bottle 2)	Between 10 and 22 hours	0.20	Fresh medium added to bacterial culture occasionally
8	Same as container 7	8:20	0.23	--

1/ Time of cessation of opercular movement.
2/ None indicates that death did not occur during the 24-hour test period.

was used in addition to these two bottles of material, which were $4\frac{1}{2}$ months old at this time. These three materials were tested in duplicate (containers 1 through 6). The test materials in all of these containers, except one of sea water (2), were sampled for bacterial counts just before the fish were added. These samples were refrigerated 20 minutes to $2\frac{1}{2}$ hours before pouring the plates.

Five containers of the test material (containers 7 through 11) were used to test the effects of heating and filtration on the toxicity of unialgal G. brevis cultures. Bacterial counts were not made for these materials because such information was not needed. A filtrate which was prepared by passing G. brevis culture through filter paper (Whatman No. 42), was tested in duplicate. A single container of another test material consisted of the residues retained by the two filter paper discs eluted in 2 liters of sea water. Two liters of G. brevis culture were passed through each disc. Other test materials included single containers of G. brevis cultures which had been heated to 35 and 45° C.

Each of the 11 containers (4-liter beakers) received two common killifish (Fundulus grandis), 3 to $3\frac{1}{2}$ inches long. The test materials, 2 liters in each container, were not aerated.

Only one of the four fish placed in each of the two control materials, sea water and Prorocentrum culture (bottle 2), failed to survive the 4-hour test period (Table 4). On the contrary, none of the four fish subjected to the G. brevis culture (bottle 1) were alive at the end of the test period. The "death times" were 9, 16, 100, and 130 minutes. Again, however, the G. brevis culture (bottle 1) had a greater bacterial concentration than the material in bottle 2, in spite of the addition of Prorocentrum a month earlier. The count for the former was 2.2 to 2.4 million bacteria per ml in contrast to 0.19 to 0.20 million per ml for the latter. The latter counts are quite similar to those obtained for bottle 2 in experiment 5 (Table 3).

The fish lived 20 to 100 minutes in the G. brevis culture heated to 35° C. In the culture heated to 45° C. the "death times" were only 13 and 18 minutes. Three of the four fish exposed to filtrates of a G. brevis culture survived the experimental period. The two fish subjected to the materials eluted from filter paper through which G. brevis culture had passed died in 23 and 130 minutes. Filtration appears to reduce the toxicity of G. brevis cultures. However, other filtering methods must be tested before this effect can be established as a characteristic of filtration.

Table 4.--Effects of unialgal cultures of G. brevis and Prorocentrum sp. on Fundulus grandis, and effects of heating and filtration on the toxicity of unialgal G. brevis cultures--experiment 6

Container No.	Material in container	Death time[1] (hr:min)	No. bacteria per ml (millions)	Remarks
1	Centrifuged aged sea water (aged 1 month in dark)	3:10 none	0.12	--
2	Same as container 1	none[2] none	--	--
3	4½-month-old G. brevis culture (bottle 1), 0.7 million organisms per liter	0:09 0:16	2.4	Fresh medium added to G. brevis culture occasionally
4	Same as container 3	1:40 2:10	2.2	--
5[3]	1-month-old Prorocentrum sp. culture (bottle 2) 0.9 million organisms per liter	2:45 none	0.20	Fresh medium added to Prorocentrum culture occasionally
6	Same as container 5	none none	0.19	--
7[4]	Filtrate (No. 42 Whatman paper) of 9-month-old G. brevis culture, 1.2 million organisms per liter	2:45 none	--	Fresh medium added to G. brevis culture occasionally. Filtrate refrigerated 2 days
8	Same as container 7	none none	--	--
9	2 liters sea water (same as in containers 1 & 2) plus filter paper discs used for containers 7 & 8. 2 liters of G. brevis culture passed through each disc	0:23 2:10	--	Filter paper discs refrigerated in dry beaker 2 days, eluted with sea water just before fish added
10	Same G. brevis culture used in preparing filtrates for containers 7 & 8--heated to 35° C., then cooled to room temperature	0:20 1:40	--	No live G. brevis observed after culture heated
11	Same as container 10 except portion of culture heated to 45° C.	0:13 0:18	--	No live G. brevis observed after culture heated

1/ Time of cessation of opercular movement.
2/ None indicates that death did not occur during the 4-hour test period.
3/ The G. brevis-free bacterial culture (bottle 2) was inoculated with unialgal Prorocentrum sp. 1 month prior to use.
4/ Another G. brevis culture was used for the heating and filtration studies because of insufficient culture in bottle 1.

Experiment 7. Comparison of Toxicity of Unialgal *Gymnodinium brevis*, *Prorocentrum* sp., and *Gymnodinium* sp., and Effects of Filtration on Toxicity

The final toxicity study in this series was performed to compare the effects of unialgal cultures of *G. brevis*, *Prorocentrum* sp., and *Gymnodinium* sp. The two latter organisms were isolated from water samples taken in the lagoon, Galveston, Texas. *Gymnodinium* sp. is morphologically similar to the cultured forms of *G. brevis* originally isolated from Florida waters. A portion of the experiment was to determine whether passage of *G. brevis* cultures through a millipore membrane would reduce toxicity as did passage through filter paper.

Striped mullet (*Mugil cephalus*) and variegated minnows (*Cyprinodon variegatus*) were used as test fish. The *M. cephalus* (3 to 4 in. long) were maintained in aerated aquaria about 24 hours before beginning the experiment. The *C. variegatus* (about $1\frac{1}{4}$ in. long), collected 3 days prior to beginning of experiment, were kept in a non-aerated aquarium since this species survives well without aeration.

Five of the seven different test materials included in this study were tested in duplicate. These materials consisted of: two different unialgal *G. brevis* cultures (containers 1 and 2, 3 and 4); *Gymnodinium* sp. (containers 5 and 6); *Prorocentrum* sp. (containers 7 and 8); and culture medium from bottle 3 (containers 9 and 10). Also included was a filtrate prepared by passing 1 liter of *G. brevis* culture through a millipore membrane (container 11) and the residues retained with this membrane eluted in 1 liter of culture medium from bottle 3 (container 12). The millipore membrane (HA) retains particles as small as 0.5 micron. Each of the 12 containers (2-liter beakers) received approximately 1 liter of test material which was not aerated.

Before adding the fish, bacterial samples were taken from two containers of *G. brevis* culture (1 and 2) and one container of culture medium (9). These containers were arbitrarily selected in order to compare the bacterial counts in come of the containers before adding the fish with those obtained after death of the test fish. The samples for bacterial counts were refrigerated from 3 to 3-2/3 hours before preparing pour-plates. All containers with dinoflagellates (1 through 8) as well as the filtrate of the *G. brevis* culture (container 11) were sampled for counts of these organisms just before adding the fish. These counts were completed within 3 hours after collection of samples.

One *M. cephalus* was placed in each of the 12 containers. Each *M. cephalus* was removed from its container shortly after death and a *C. variegatus* was added. The fish were not introduced simultaneously since the *M. cephalus* were rather large for the containers.

Immediately after the M. cephalus died in the containers which were initially sampled for bacterial counts (1, 2, and 9), these containers were again sampled for such counts. Such samples were also taken from one of each of the remaining duplicate test materials (containers 3, 5, and 7) and from the millipore filtrate (container 11) following the death of the M. cephalus. Pour-plates were prepared with these samples after 4 to 6½ hours refrigeration, except the sample from container 7 which was stored only 1-1/3 hours.

The results (Table 5) of this 27-hour experiment show that all of the M. cephalus subjected to either G. brevis cultures or filtrate and residues of such a culture died in less than an hour. The "death times" varied from 14 to 53 minutes. The M. cephalus in the uninoculated culture medium lived approximately 2-1/3 and 3 hours. The bacterial count of 1.8 million per ml of this culture medium (bottle 3) was higher than the 1.0 to 1.5 million per ml obtained for the G. brevis cultures. The M. cephalus in the Prorocentrum culture survived nearly 7 hours. Those exposed to the Gymnodinium sp. died after 46 and 69 minutes.

The C. variegatus survived considerably longer than the M. cephalus in all test materials. In the G. brevis cultures the "death times" for C. variegatus varied from 2-2/3 to 7-8 hours. This species survived the 27-hour test period in the two control materials (the culture medium and the Prorocentrum culture). The C. variegatus lived about 2-1/3 and 2-2/3 hours in the Gymnodinium sp. culture.

M. cephalus lived only 14 minutes in the millipore filtrate of the G. brevis culture in contrast to 53 minutes in material eluted from the millipore membrane. Likewise the filtrate was more toxic to C. variegatus than the residues; the fish lived 4½ hours in the former material whereas the one in the latter survived the test period.

Discussion of Results of Experiments with Unialgal Cultures

The fish subjected to unialgal cultures of G. brevis died more rapidly than those exposed to control materials in the seven experiments considered in this section, excepting one fish. The greater survival of the control fish demonstrates that unialgal cultures of this organism are toxic to the five species of fish tested (Membras vagrans, Mugil cephalus, Fundulus grandis, Cyprinodon variegatus, and Mollienisia latipinna). Indeed, the rapidity with which the fish succumbed in some of the cultures emphasizes the toxicity of unialgal G. brevis cultures. For example, the minimum "death times" for some of these fish were: 4 minutes for M. vagrans (only one tested), 14 to 16 minutes for M. cephalus, and 9 to 16

Table 5.--Effects of unialgal cultures of G. brevis, Prorocentrum sp., and Gymnodinium sp. on Mugil cephalus and Cyprinodon variegatus, and effects of filtration on the toxicity of unialgal G. brevis cultures---experiment 7

Container No.	Material in container	Distress time1/ M. cephalus	Death time2/ M. cephalus	Distress time1/ C. variegatus	Death time2/ C. variegatus	No. dinoflagellates per liter (millions)3/	No. bacteria per ml (millions)4/
1	5-week-old G. brevis culture	0:39	0:49	4:38	5:20	1.3	1.5 (1.3)
2	Same as container 1	0:10	0:16	not known	7-8 hours	1.3	1.0 (1.1)
3	1-year-old G. brevis culture, fresh medium added 6-8 weeks prior to use	0:10	0:16	2:39	2:42	1.2	--- (1.1)
4	Same as container 3	0:09	0:17	2:42	6:04	1.0	---
5	6-week-old Gymnodinium sp. culture	0:32	1:09	2:10	2:23	1.4	--- (0.90)
6	Same as container 5	0:22	0:46	2:23	2:42	1.1	---
7	8-month-old Prorocentrum sp. culture, fresh medium added approximately weekly	not known	6:52	none5/	none	1.0	--- (3.3)
8	Same as container 7	not known	6:51	none	none	0.9	---
96/	6-week-old culture medium (uninoculated) from reservoir (bottle 3)	2:13	2:20	none	none	---	1.8 (2.1)
10	Same as container 9	2:54	3:05	none	none	---	---
11	Millipore filtrate of portion of same culture used in containers 3 & 4	0:06	0:14	3:26	4:30	0	--- (0.0060)
12	Millipore membrane used to obtain filtrate in container 11 eluted in 1 liter culture medium from bottle 3 (same as used in containers 9 & 10)	0:48	0:53	none	none	---	---

1/ Time (hr:min) required for fish to show first signs of imbalance.
2/ Time (hr:min) of cessation of opercular movement.
3/ All samples for dinoflagellate counts were collected from the containers just before introducing the fish.
4/ Samples for the first bacterial count listed for each container were collected just before introducing the M. cephalus; samples for the second counts, enclosed in parentheses, were taken immediately after the M.cephalus died.
5/ None indicates that death or distress did not occur during the 27-hour test period.
6/ Original supply of medium in reservoir (bottle 3) became exhausted and it was renewed about 6 weeks prior to use.

minutes for F. grandis. However, all five species did not show such extremes of sensitivity to G. brevis cultures. The minimum "death times" for C. variegatus were 2-1/3 to 2-2/3 hours.

The concentration of G. brevis in the cultures used for the seven experiments varied from 0.6 to 2.1 million per liter. Since these cultures were not free of bacteria, such organisms possibly contributed to their toxicity.

The results of a preliminary study suggest that the survival period after exposure to G. brevis depends on the exposure time and that fish subjected for just a few minutes may not recover when transferred to sea water. M. cephalus, subjected to unialgal G. brevis for 3 and 15 minutes and then transferred to sea water, lived for 165 and 12 minutes, respectively (Experiment 5).

Moreover, there is some suggestion that G. brevis cultures may become more toxic with each subsequent addition of test fish. For example, in experiment 5, the "death times" of each of four M. cephalus added in succession to the same culture decreased progressively from 29 to 7 minutes. We do not know the reason(s) for this apparent increase in toxicity. Since the test materials were not aerated, possibly the oxygen content was progressively lowered by the test fish and thereby decreased the "death times".

The toxicity of unialgal G. brevis cultures does not depend on the presence of living organisms. Cultures heated to 35 and 45° C. were no less toxic than the untreated ones. The removal of G. brevis from a culture by millipore filtration did not reduce the toxicity. The relative toxicity of a filtrate appears to be dependent upon the type of filter membrane employed. A paper membrane (Whatman No. 42) apparently retains the more toxic portion of the culture.

Attempts to determine whether G. brevis or its associated bacterial flora produces the toxic substance were not entirely successful. G. brevis-free cultures presumed to contain the bacterial flora associated with unialgal cultures of this organism were not toxic to the test fish. However, when these experiments were performed, the bacterial counts of these cultures were considerably lower than those of the G. brevis cultures. Nevertheless, a culture of presumed associated bacteria with a count of slightly more than a million per ml proved non-toxic to M. cephalus. The bacterial count in this culture of experiment 4 (Table 2) was comparable to those of the unialgal cultures which were toxic to M. cephalus and C. variegatus in experiment 7 (Table 5). Furthermore, in the latter experiment uninoculated culture medium containing about 2 million bacteria per ml, which was slightly higher than the counts of two different G. brevis cultures, was not toxic to the test fish.

Unialgal cultures of three species of dinoflagellates (Gymnodinium splendens, Gymnodinium sp., and Prorocentrum sp.) isolated from the lagoon, Galveston, Texas, were tested for toxicity to fish. Two species were non-toxic and one proved toxic. The non-toxic forms, G. splendens and Prorocentrum sp., were used in concentrations comparable to and, in some cases, even exceeding those of the toxic G. brevis. In fact, M. cephalus survived considerably longer in the Prorocentrum culture than in the control material (uninoculated culture medium) in experiment 7 (Table 5). The Prorocentrum possibly aided survival of the fish by liberating oxygen since the test materials were not aerated. The Gymnodinium sp. was toxic to M. cephalus and C. variegatus (Table 5). In view of these results and since this organism in culture is morphologically similar to G. brevis, we tentatively consider the Galveston Gymnodinium to be G. brevis. The Galveston Gymnodinium was observed only three times (all within the same week) although samples were collected from the lagoon three times weekly during an 11-month period. The concentrations in the lagoon samples varied form 1,000 to 60,000 organisms per liter.

EXPERIMENTS WITH BACTERIA-FREE CULTURES OF GYMNODINIUM BREVIS

Mass bacteria-free cultures of G. brevis were established following the completion of experiment 7. The two experiments to follow (8 and 9) were the first toxicity tests to be conducted with pure cultures. The importance of these studies lies in the fact that the observed effects can be attributed to G. brevis with certainty since no other organisms were present during the incubation period. Substantiation of the toxicity of unialgal G. brevis with bacteria-free G. brevis should establish the existence of a cause and effect relationship between "blooms" of this organism and associated mass mortality of marine animals. The experiments with bacteria-free cultures were more refined in several respects than those with unialgal cultures. In addition to aerating most of the test materials, such factors as temperature, dissolved oxgen, salinity, and pH of these materials were determined.

Experiment 8. Effects of Unialgal and Bacteria-free Gymnodinium brevis Cultures

The first mass bacteria-free cultures of G. brevis were tested for toxicity to striped mullet (Mugil cephalus) and variegated minnows (Cyprinodon variegatus). The C. variegatus ($1\frac{1}{4}$ to $1\frac{1}{2}$ in. long) and M. cephalus (1 to $1\frac{1}{4}$ in. long) were maintained in aerated aquaria for 5 days and overnight, respectively, before beginning of experiment. One liter of distilled water was placed in each 2-liter beaker, containers for the various test materials, 5 days before commencing the experiment so that the aeration equipment could be tested and adjusted. The water was discarded and the beakers received no further treatment before introducing the various test materials.

The material in 12 of the 14 containers received gentle aeration continously from a small aerator; the main air line from the aerator was equipped with a non-absorbent cotton filter. To preclude possible excessive oxygen demand by G. brevis, light was provided continously with two fluorescent lamps equipped with two 18-inch, 15-watt daylight tubes. A photograph of the experimental setup is presented in figure 1.

Six different bacteria-free cultures (containers 3, 4, 6, 7, 9, 10, 12, and 13) and four batches of sterile control material (containers 5, 8, 11, and 14), all of which had incubated for a month, were employed in this study. The control material consisted of uninoculated culture medium otherwise subjected to the same conditions as the inoculated medium. The sterility of the G. brevis cultures and control materials was established by inoculating routine sterility-test media with samples withdrawn from the culture vessels shortly before these materials were dispensed into the experimental containers. Duplicate containers of two of the six bacteria-free cultures (3 and 4, 6 and 7) were set up to compare the survival of test fish in aerated (containers 3 and 6) and non-aerated (containers 4 and 7) cultures. A year-old unialgal G. brevis culture (container 1), which was replenished with fresh medium about 4 days previously, was used to compare the effects of unialgal and bacteria-free cultures. Aged sea water (container 2) served as control material for the entire experiment. The volume of test material placed in each container varied from 750 to 1000 ml.

Before adding the fish, samples were taken from four containers of bacteria-free G. brevis culture (3,6,9, and 12) and two containers of uninoculated culture medium (5 and 11) for bacterial counts. Also at this time the nine containers with G. brevis were sampled for enumeration of this organism. The samples taken for the initial bacterial counts were refrigerated from 1 to nearly 3 hours before preparing the pour-plates. Most of the G. brevis counts were completed within a few minutes to an hour after withdrawing the samples. A few samples, however, stood for a maximum of approximately 3 hours.

Each of the 14 containers received two M. cephalus and two C. variegatus except the container of sea water (2), which received three M. cephalus. Each fish was removed from the container shortly after it died so that test materials would not become excessively fouled by decomposing fish.

After commencing the experiment, samples were again taken for G. brevis and bacterial counts. Seven of the containers with G. brevis were sampled for counts of this organism immediately after the death of the last fish in the container. Two other containers of G. brevis (12 and 13), in which the last fish in the container

survived beyond 8 hours, were sampled about 8 hours after beginning the experiment. The second samples for bacterial counts were taken either immediately after the death of the last fish in the container or after 30 to 31½ hours providing one fish survived the test period (31½ hours). The 10 containers sampled for bacterial counts included all of those which were sampled for such counts initially (3, 5, 6, 9, 11, and 12), the container of unialgal G. brevis (14), the container of sea water (2), and the two containers of non-aerated G. brevis cultures (4 and 7). These samples were plated after 15 to 90 minutes refrigeration.

Following the collection of bacterial and G. brevis samples, all test materials were sampled for dissolved oxygen, pH, and salinity determinations (Table 7). Aeration of each container (if aerated) was discontinued just before collecting the samples, which were taken either immediately after the death of the last fish in the container or near the end of the experimental period if at least one test fish survived. Seven containers (2, 5, 8, 11, 12, 13, and 14), those in which at least one fish survived beyond 7 hours, were also sampled for pH determinations 7 to 7½ hours after beginning the study. During this 31½-hour experiment the room temperature varied from 20 to 24.5° C.

Only one of the 32 fish subjected to initially bacteria-free cultures survived the 31½-hour test period; all except three (C. variegatus) died within 8 hours (Table 6). The lone fish (C. variegatus) surviving the experimental period succumbed about 30 minutes later. On the contrary, only one of the 21 fish exposed to control materials of sea water and initially sterile culture medium failed to survive the test period. This fish (C. variegatus) died after nearly 30 hours in culture medium. The "death times" of M. cephalus, which varied from ¼ to 4-3/4 hours, were considerably less than those of the C. variegatus, 3¼ to nearly 32 hours. Fifty percent (16) of the test fish in the bacteria-free cultures died earlier than those (4) in the unialgal culture; the "death times" in this culture were about 1¼ hours for M. cephalus and 6¼ and 6-3/4 hours for C. variegatus. The test fish survived considerably longer in the non-aerated than in the aerated G. brevis culture in one instance; in the other case the opposite occurred although the differences of survival in the two types of cultures were less marked.

The bacterial counts of the initially bacteria-free G. brevis cultures sampled before adding the fish varied from 250 to 20,000 per ml. The rather high initial bacterial counts are attributed to the prolonged standing (5 days) of the distilled water in the experimental containers while the aeration equipment was being tested and adjusted. The counts obtained from these cultures following the death of the last fish in the container (5 to 8

Table 6.--Effects of bacteria-free and unialgal cultures of G. brevis on Mugil cephalus and Cyprinodon variegatus---experiment 8

Container No.	Material in container[1]	Distress time[2] M. cephalus	Death time[3] M. cephalus	Distress time[2] C. variegatus	Death time[3] C. variegatus	No. G. brevis per liter (millions)[4]	No. bacteria per ml (millions)[4]
	1000 ml unialgal G. brevis	1:06	1:13	5:55	6:17	3.4	---
1	culture, pH 8.0	1:07	1:13	not known	6:47	(2.4)	(3.5)
2	1000 ml aged sea water	none[5]	none	none	none	---	--- (5.0)
	1000 ml bacteria-free G. brevis	0:13	0:19	3:10	3:18	4.1	0.00025
3	culture	0:19	0:29	4:55	5:33	(3.7)	(0.033)
	1000 ml of same culture used	1:34	1:44	5:42	5:47	3.1	---
4	in container 3, but not aerated	1:39	1:44	5:44	5:56	(2.0)	(0.075)
	1000 ml sterile culture medium,			6:50	29:48		0.0010
5	pH 7.6---control for 3 & 4	none	none	none	none	---	(21.0)
	800 ml bacteria-free G. brevis	1:07	1:47	7:20	7:47	4.8	0.020
6	culture	1:38	1:48	7:22	7:50	(2.6)	(0.30)
	800 ml of same culture used in	0:28	0:34	5:10	6:05	4.0	---
7	container 6, but not aerated	0:28	1:56	6:31	7:02	(3.2)	(0.60)
	800 ml sterile culture medium,						
8	pH 7.6---control for 6 & 7	none	none	none	none	---	---
	1000 ml bacteria-free G. brevis	0:10	0:15	3:40	3:51	3.2	0.0070
9	culture, pH 7.7	0:12	0:25	4:55	4:58	(2.7)	(0.075)
	1000 ml bacteria-free G. brevis	0:13	1:09	3:21	3:44	3.5	
10	culture, pH 7.7	0:13	1:12	3:48	5:54	(2.4)	---
	1000 ml sterile culture medium,						0.025
11	pH 7.7---control for 9 & 10	none	none	none	none	---	(7.0)
	750 ml bacteria-free G. brevis	4:18	4:24	6:07	27:17	3.4	0.0050
12[6]	culture	4:32	4:44	31:27	31:57	(1.8)	(24.0)
	750 ml bacteria-free G. brevis	2:16	2:26	7:14	7:58	2.3	
13	culture	2:19	2:41	7:40	15:08	(1.5)	---
	750 ml sterile culture medium						
14	---control for 12 & 13	none	none	none	none	---	---

1/ Determinations of pH made on samples withdrawn directly from culture vessel. All containers aerated except as noted.
2/ Time (hr:min) required for fish to show first visible signs of imbalance.
3/ Time (hr:min) of cessation of opercular movement.
4/ The first G. brevis and bacterial counts listed for each container were obtained from samples colected before introduction of the fish. Samples for second counts, enclosed in parentheses, were taken immediately after the death of last fish in container, except for two G. brevis counts (containers 12 and 13) and four bacterial counts (containers 2, 5, 11, and 12); the former were taken about 8 hours and the latter 30 to 31½ hours after start of experiment.
5/ None indicates that distress or death did not occur during the 31½-hour test period.
6/ The last fish (C. variegatus) in container 12 died about 30 minutes after close of experiment.

Table 7.--Dissolved oxygen, temperature, salinity, and pH data for test materials of experiment 8

Container No.[1]	Dissolved oxygen (ppm)	Dissolved oxygen (% saturation)[2]	Temperature[3] (°C)	Salinity (o/oo)	pH[4]	Time[5] (hr)
1	6.77	89.4	22.8	29.84	7.5	7
2	6.10	85.2	23.6	35.93	(7.6) 7.4	30-3/4
3	6.70	89.3	21.9	32.73	7.3	5½
4	3.38	45.0	22.1	32.72	7.2	6
5	5.69	77.8	23.8	32.66	(7.2) 7.2	30-1/3
6	6.02	81.7	23.0	33.43	7.3	7½
7	4.18	56.7	22.8	33.86	7.4	7
8	5.59	76.8	23.8	33.44	(7.2) 7.2	30-3/4
9	6.48	86.1	21.7	33.80	7.4	5
10	6.84	91.5	22.4	32.71	7.4	6
11	6.62	90.8	23.7	32.97	(7.3) 7.3	31
12	6.60	90.6	23.9	32.69	(7.3) 7.4	31
13	6.34	86.0	23.3	32.71	(7.3) ---	15
14	6.10	83.7	23.8	32.97	(7.1) 7.2	31¼

1/ See table 6 for identity of materials in the containers.
2/ Percentage of saturation in relation to sea water of the given temperature and salinity in equilibrium with normal dry atmosphere.
3/ Temperature of material in container at time dissolved oxygen was determined.
4/ All pH values enclosed in parentheses were determined 7 to 7½ hours after start of experiment.
5/ The approximate time which elapsed between the start of experiment and collection of samples for analyses.

hours later) varied from 33,000 to 600,000 bacteria per ml. A count of 24 million bacteria per ml was obtained after 31½ hours from the initially bacteria-free G.brevis culture in which one fish survived the test period. The first bacterial counts for two containers of initially sterile culture medium were 1,000 (container 5) and 25,000 (container 11) per ml. When these containers were sampled again near the end of the 31½-hour test period the bacteria had increased to 21 (container 5) and 7 (container 11) million per ml.

The results of experiment 8 show clearly that bacteria-free G. brevis cultures are toxic to fish. Nevertheless, we desired to confirm this toxicity using test materials with greatly reduced initial and terminal bacterial counts.

Experiment 9. Effects of Bacteria-free and Unialgal Gymnodinium brevis Cultures, and Effects of Filtration on Toxicity

The second study with bacteria-free G. brevis differed somewhat from the first one (Experiment 8). In experiment 9 the initial bacterial contamination of test materials by containers and aeration equipment was reduced; the effects of two methods of filtration on the toxicity of bacteria-free cultures were studied; and the sensitivity of two size groups of mullet (Mugil cephalus) to G. brevis cultures was compared. The experimental setup was the same as for experiment 8 (Fig. 1) except that more containers and a larger air pump were used.

We employed three precautions to reduce the initial bacterial contamination of the test materials. One precaution was heating the experimental containers (2-liter beakers) in a hot air oven at 150-160° C. for 2 hours and allowing them to cool overnight in the oven. The containers were removed from the oven shortly before the test materials were added. Secondly, the aeration apparatus was autoclaved for 15 minutes at 15 pounds pressure. Before sterilization this apparatus was assembled and packaged so that a glass air-delivery tube could be inserted into each of the 18 containers without handling the portion which contacted the test materials. All air pumped into the containers passed through a non-absorbent cotton filter installed in the main air line from the pump. The third safeguard against excessive bacterial contamination was placing the test fish in autoclaved 85% aged sea water for several minutes before transferring them to the experimental containers. This concentration of sea water is about the same as that in the culture media to which the fish were exposed.

Three different month-old bacteria-free G. brevis cultures, two batches of sterile culture medium of the same age, a 10-week-old unialgal G. brevis culture, and autoclaved 85% aged sea water constituted the test materials. One liter of test material was placed in

each of the 18 containers. Since the test materials were aerated more vigorously in this study, the increased air flow made equalizing the degree of aeration in each container difficult and the agitation of the test materials probably varied more.

One portion of the experiment (containers 1 through 8) was designed to compare the effects of millipore (HA membrane) and paper (Whatman No. 40) filtration on the toxicity of one of the bacteria-free G. brevis cultures. One batch of the sterile culture medium treated in the same manner as the G. brevis culture served as control material. The millipore and paper residues of both the G. brevis culture and the sterile culture medium were each eluted in 1 liter of sterile culture medium to obtain four of the eight test materials used in this phase of the study. The test materials for the remaining portion of this experiment (containers 9 through 18) consisted of duplicate containers of two different bacteria-free G. brevis cultures, sterile culture medium, autoclaved 85% aged sea water, and a unialgal G. brevis culture. The distribution of the test materials and numerical designation of the containers are presented in table 8.

Samples were taken for bacterial and G. brevis counts immediately after dispensing the test materials. All containers of unfiltered bacteria-free G. brevis culture (9, 10, 11, and 12) and unfiltered culture medium (13 and 14) in addition to a container of sea water (15) and unialgal G. brevis culture (17) were sampled for bacterial counts. None of the containers of filtrates or residues were sampled because such counts were not needed. These samples were refrigerated $4\frac{1}{2}$ to $6\frac{1}{2}$ hours before plating. Samples for G. brevis counts were obtained from the containers with filtrates of G. brevis culture (1 and 2) and bacteria-free G. brevis (9, 10, 11, 12). The G. brevis concentration of the unialgal culture used in containers 17 and 18 was ascertained by withdrawing a sample from the culture prior to dispensing. The G. brevis samples were counted between $1\frac{1}{2}$ to 2-1/3 hours after collection.

Each container received four fish: Three small mullet (1 to $1\frac{1}{4}$ inch long) and one large mullet ($4\frac{1}{2}$ to $5\frac{1}{2}$ inches long). The small mullet were maintained in aerated aquaria overnight and the large mullet were used within a few hours after collection. The volume of test material (1 liter) was somewhat small for some of the large mullet, which thrashed about vigorously. This activity possibly injured some of the smaller test fish.

Excepting the filtrates of G. brevis cultures (containers 1 and 2), the bacteria and G. brevis were again enumerated for those containers for which such counts were made initially. The second bacterial samples from the five containers with G. brevis (9, 10, 11, 12, and 17) were plated after 4-3/4 to $5\frac{1}{4}$ hours refrigeration whereas those from the containers with culture medium (13 and 14) and sea water (15) were stored only 2/3 to $1\frac{1}{4}$ hours. The second

G. brevis counts were completed between 1 and 2 hours after collecting the samples. In addition, samples for pH and salinity determinations were taken from all 18 containers. Bacterial, G. brevis, pH, and salinity samples were taken either shortly after the death of the last fish in the container or at the end of the test period (24 hours) providing one fish in the container survived, except for some pH and salinity samples as noted under "remarks" in table 8. The room temperature varied from 22 to 25° C. during the 24-hour test period.

The fish subjected to bacteria-free cultures as well as filtrates and residues of such cultures died more rapidly for the most part than those exposed to the control materials (Table 8). Some of the control fish, especially small M. cephalus, did not survive well. Fish of this size group died rapidly in one batch of sterile culture medium (containers 13 and 14). The "death times" varying from 25 minutes to 2-2/3 hours for the small mullet in these two containers were comparable to the "death times" varying from 15 minutes to $2\frac{1}{2}$ hours for the same sized fish in unfiltered bacteria-free and unialgal G. brevis cultures. However, the large M. cephalus in the two containers of culture medium survived considerably longer than those in the G. brevis cultures. One large fish survived the 24-hour test period and the other lived at least $8\frac{1}{2}$ hours. In contrast, the "death times" for the six large mullet in three different unfiltered G. brevis cultures, two of which were bacteria-free, varied from 10 to 41 minutes. All fish in the sea water lived for at least $7\frac{1}{2}$ hours and two of them survived the test period.

The early deaths of the small mullet in containers 13 and 14 were probably due to the abnormally low pH of the culture medium. The pH of the material in container 14 was 5.9 about 14 hours after beginning of experiment and increased to 6.4 after 25 hours. A pH value of 6.6 was obtained for the culture medium in container 13 at the end of the test period.

The control fish lived considerably better in the batch of sterile culture medium used in the filtration phase of this experiment. All of the large mullet (4) and two-thirds of the small mullet (8) subjected to filtrates and residues of culture medium survived the 24-hour test period. On the contrary, none of the fish (4 large mullet and 12 small mullet) exposed to filtrates and residues of the G. brevis culture survived the test period. The difference between the effects of the two methods of filtration on the toxicity of the G. brevis culture was marked. The millipore filtrate was much more toxic to the fish than the residues; in the filtrate the "death times" varied from 14 to 104 minutes in contrast to a variation of 5 to $13\frac{1}{2}$ hours in the residues. The toxicity of the filter paper residues was greater than the filtrate; the test fish lived from 39 to 42 minutes in the residues whereas they survived from 44 minutes to about 8 hours in the filtrate.

Table 8.--Effects of bacteria-free and unialgal G. brevis cultures on two size groups of Mugil cephalus, and effects of filtration on the toxicity of bacteria-free cultures--experiment 9

Container No.	Material in container	Distress time[1]	Death time[2]	No. G. brevis per liter (millions)[3]	No. bacteria per ml (millions)[3]	pH[3]	Salinity[2]	Remarks
1	Millipore filtrate (HA membrane) of 1 liter of bacteria-free G. brevis culture, 3.3 million organisms per liter. Two membranes used, first one became clogged.	(0:09) 0:19 0:42 1:10	(0:14) 0:25 1:27 1:44	0	--	7.7	33.3	Salinity--2/3 hour after last fish died
2	Filtrate (paper, Whatman No. 40) of 1 liter of the same culture which was used to prepare the filtrate in container 1.	(0:46) 0:34 0:44 0:55	(0:54) 0:44 1:10 8:05	0.0080	--	7.5	33.0	Some G. brevis passed by the filter paper when it was inadvertently overflowed
3	Both millipore membranes used to prepare filtrate in container 1 eluted in 1 liter of sterile culture medium, which was 1 week old with a pH of 7.8	(not known) not known 5:00 5:05	($8\frac{1}{2}$-$13\frac{1}{2}$ hr) 5:00 5:06 5:10	--	--	7.5	30.7	Salinity and pH--after 14 hours
4	Filter paper disc used to prepare the filtrate in container 2 eluted in 1 liter of sterile medium from same batch as used for elution in container 3.	(0:31) 0:36 0:37 0:38	(0:39) 0:42 0:42 0:42	--	--	6.8	28.9	pH--2/3 hour and salinity--1-1/3 hours after last fish died
5	Millipore filtrate (HA membrane) of 1 liter of sterile culture medium--control for container 1.	(none)[4] 0:48 2:12 none	(none) 2:01 2:17 none	--	--	7.2	33.2	--
6	Filtrate (paper, Whatman No. 40) of 1 liter of sterile medium from same batch as used to prepare filtrate in container 5--control for container 2.	(none) not known none none	(none) 1:13 none none	--	--	7.0	33.4	--
7	Millipore membrane used to prepare filtrate in container 5 eluted in 1 liter of sterile medium from same batch as used for elution in container 3--control for container 3.	(none) 0:41 none none	(none) 0:53 none none	--	--	7.1	32.9	--
8	Filter paper disc used to prepare the filtrate in container 6 eluted in 1 liter of sterile medium from same batch as used for elution in container 3--control for container 4.	(none) none none none	(none) none none none	--	--	7.1	30.7	--

1/ Time (hr:min) required for fish to show first signs of imbalance. The first distress time listed for each container, enclosed in parantheses, pertains to large mullet ($4\frac{1}{2}$ to $5\frac{1}{2}$ in. long) and the other three distress times pertain to small mullet (1 to $1\frac{1}{4}$ in. long).

2/ Time (hr:min) of cessation of opercular movement. The first death time listed for each container, enclosed in parentheses, pertains to large mullet and the other three death times pertain to small mullet.

3/ The first G. brevis and bacterial counts listed for each container were obtained from samples taken from the containers before adding the fish except for containers 17 and 18. For these containers, the sample was withdrawn from the culture before portions were dispensed into containers 17 and 18. With the exceptions noted under "remarks", samples for the second G. brevis and bacterial counts, those enclosed in parentheses, as well as pH and salinity samples were collected either shortly after the last fish died if all fish died within 24 hours or at the end of the 24-hour experimental period if at least one fish survived.

4/ None indicates that distress or death did not occur during the 24-hour test period.

Table 8.--Continued[5]

Container No.	Material in container	Distress time[1]	Death time[2]	No. G. brevis per liter (millions)[2]	No. bacteria per ml (millions)[2]	pH[3]	Salinity[3]	Remarks
9	Bacteria-free G. brevis culture	(0:15) 0:16 0:23 0:32	(0:23) 0:42 1:45 1:54	2.4 (2.3)	0 (0.0050)	7.6	33.0	Salinity--½ hour after last fish died
10	Same as container 9	(0:16) 0:32 0:48 0:58	(0:28) 1:41 2:14 2:28	3.1 (2.9)	0.000008 (0.015)	7.3	32.8	--
11	Bacteria-free G. brevis culture	(0:30) 0:42 0:43 0:43	(0:41) 0:44 0:45 1:08	2.7 (2.8)	0.000001 (0.030)	7.1	33.0	Salinity--1-3/4 hours after last fish died
12	Same as container 11	(0:32) 0:35 0:37 0:41	(0:40) 0:45 0:45 0:45	3.3 (1.8)	0.00010 (0.0044)	7.1	33.1	Salinity--1½ hours after last fish died
13	Sterile culture medium--control for containers 9, 10, 11, and 12	(none) 0:18 0:30 0:52	(none) 0:25 0:33 1:38	--	0.000002 (in excess of 80 millions)	6.6	33.3	--
14	Same as container 13	(not known) 0:46 0:52 not known	(8½-13½ hr) 0:47 0:56 2:39	--	0.000025 (25 to 50 millions)	5.9 (6.4)	33.3	Salinity, first pH, and second bacterial count--after 14 hours. Second pH (6.4)--after 25 hours
15[6]	Autoclaved 85% sea water	(not known) not known not known not known	(14:10) 8½-13½ hr 8½-13½ hr 8½-13½ hr	--	0.000012 (in excess of 80 millions)	7.3	33.0	Salinity, pH, and second bacterial count--after 14½ hours
16	Same as container 15	(not known) not known none none	(8½-13½ hr) 7:16 none none	--	--	7.6	33.1	--
17	10-week-old unialgal G. brevis culture which was not replenished with fresh medium	(0:12) 0:24 0:54 1:48	(0:14) 0:45 0:56 2:30	2.6 (2.7)	1.3 (1.4)	7.6	33.2	--
18	Same as container 17	(0:06) 0:12 0:14 0:24	(0:10) 0:15 0:20 0:25	2.6	--	7.9	33.2	pH--1 hour and salinity--2 hours after last fish died

5/ See page 29 for footnotes 1, 2, 3, and 4.
6/ The large mullet in container 15 probably died of oxygen deficiency. The fish appeared in good condition after 13½ hours and the material in the container was being aerated. When noticed again 30 minutes later the fish was dying and no air was being pumped in the container at this time.

The attempt to reduce the initial bacterial contamination of the bacteria-free G. brevis cultures and the sterile culture medium by the previously mentioned precautions was successful. The counts for these materials varied from 0 to 100 bacteria per ml. The bacterial counts in the initially bacteria-free G. brevis cultures varied from 4,400 to 30,000 per ml at the time the last fish died in the container (3/4 to 2½ hours later). The initial count in the unialgal culture was 1.3 million bacteria per ml; when the last fish died 2½ hours later the count was 1.4 million per ml. At the end of the test period the bacterial concentration was in excess of 80 million per ml in one container of initially sterile culture medium (13). After about 14 hours the count was 25 to 50 million bacteria per ml in the other container of culture medium (14) and in excess of 80 million per ml in one container of sea water.

The pH of the initially sterile culture medium in container 14 increased from 5.9 to 6.4 during the 11-hour period after the last fish died. Additional fish were subjected to this material to test its toxicity at the higher pH.

Experiment 9a. Supplementary Toxicity Tests of Some Test Materials Previously used in Experiment 9

Near the close of experiment 9, we conduct a supplementary study to determine whether the initially sterile culture medium in container 14 of experiment 9 was still toxic to small M. cephalus. Five other containers originally a part of experiment 9, one container of 85% autoclaved sea water (15) and the four containers of initially bacteria-free G. brevis culture (9, 10, 11, and 12), were included in this study. The other containers of culture medium (13) and sea water (16) were not used because this part of the experiment was still in progress as they contained one or more live fish.

Small mullet from the group used in experiment 9 served as test fish. Four of these fish, which were maintained in 85% autoclaved sea water for 24 hours, were placed in each container. Only two of the four containers of G. brevis culture were aerated in an attempt to determine the effects of agitation and aeration on the toxicity of G. brevis. The culture medium (container 14) and sea water (container 15) were not aerated.

The results (Table 9) show that G. brevis cultures were still toxic to small mullet, but the fish survived well in the previously toxic culture medium. The 16 fish in the G. brevis cultures died between 8 and 125 minutes; 10 of them survived less than 1 hour. In the control materials (containers 14 and 15), however, only one of the eight fish failed to survive the 24-hour test period; one fish in the sea water succumbed after 5-3/4 hours. Although the experiment was discontinued after 24 hours, containers

Table 9.--Effects of certain test materials initially used in experiment 9 on Mugil cephalus--experiment 9a

Container No.[1]	Distress time[2]	Death time[3]	No. G. brevis per liter (millions)	Remarks
9	0:37 1:02 1:18 1:50	1:26 1:28 2:05 2:05	0.9 (sample taken 30 minutes after fish added)	Material aerated continuously during previous 24-hour period, aerated vigorously for last 12 hours and during experimental period. Material not cloudy.
10	0:05 0:20 0:20 0:20	0:36 0:55 1:11 1:11	2.4 (sample taken 25 minutes after fish added)	Material neither aerated during previous 21-hour period nor during experimental period. Material not cloudy.
11	not known 0:08 0:10 0:18	0:08 0:18 0:18 0:34	1.3 (sample taken 15 minutes after fish added)	Material not aerated during previous 22-hour period. Vigorous aeration began 18 minutes before fish added and continued throughout experiment. Material not cloudy.
12	0:05 0:05 0:08 0:08	0:10 0:10 0:10 0:13	1.4 (sample taken 15 minutes after fish added)	Material neither aerated during previous 23-hour period nor during experimental period. Material not cloudy.
14	none[4]	none	---	Material neither aerated during previous 10-hour period nor during experimental period. Material was cloudy when fish added. Two fish developed heavy microbial growth on caudal fin, they died between 30 and 48 hours after study was begun. Two fish alive after 5 days. Material relatively clear at this time.
15	not known none none none	5:47 none none none	---	Material neither aerated during previous 9-hour period nor during experimental period. Material cloudy when fish added but relatively clear 5 days later. Three fish alive after 5 days.

1/ See table 8 (Experiment 9) for identification of container contents.
2/ Time (hr:min) required for fish to show first signs of imbalance.
3/ Time (hr:min) of cessation of opercular movement.
4/ None indicates that distress or death did not occur during the 24-hour test period.

14 and 15 were set aside and observed for 4 more days. Two of the fish in the culture medium died between 30 and 48 hours, and the other two were alive after 5 days. The three fish remaining in the sea water also lived through 5 days. There was some indication that non-aerated G. brevis cultures were more toxic than the aerated ones.

Discussion of Results of Experiments with Bacteria-free Cultures

The results of the experiments with bacteria-free G. brevis confirm that this organism produces a fish-killing substance(s) as indicated by tests with unialgal cultures. Bacteria-free G. brevis was toxic to the two species of fish tested (Cyprinodon variegatus and Mugil cephalus). The minimal "death time" for C. variegatus was about $3\frac{1}{4}$ hours. The mullet were more sensitive with minimum "death times" as low as 15 minutes. Furthermore, small mullet appear to be less sensitive than the large ones since the three small fish in each container of G. brevis culture outlived the large one. The concentration of G. brevis in these bacteria-free cultures varied from 2.3 to 4.8 million organisms per liter. Such concentrations are considerably less than the 10 to 60 million per liter sometimes encountered in areas where dead of dying fish occur.

There was good agreement between the results of experiments 8 and 9 with regard to the toxicity of bacteria-free G. brevis cultures to small M. cephalus, which was the only species tested in both experiments. Nevertheless, the small mullet survived much better in the control materials in experiment 8 then in experiment 9. In the latter experiment the small mullet died rapidly in one particular batch of sterile culture medium (containers 13 and 14). Large M. cephalus, however, survived much better in this batch of medium.

We attribute the early death of the small mullet in the culture medium placed in containers 13 and 14 of experiment 9 to the abnormally low pH of this particular batch of medium. Moreover, the relatively poor survival of these fish in some of the other control containers of experiment 9 was possibly due to their being damaged by the vigorous thrashing about of the large mullet. The length of some of the large test fish slightly exceeded the inside diameter of the experimental containers.

The low pH of the material in container 14 suggests that the batch of medium placed in container 14 was abnormal before the fish were added. Approximately 14 hours after experiment 9 was begun the pH of the medium in container 14 was 5.9 and the bacterial count was between 25 and 50 million per ml. Since the pH of this material increased from 5.9 to 6.4 during the next 11 hours in spite of heavy bacterial growth, the initial pH of this

particular batch of sterile medium possibly was lower than 5.9. The pH of the medium in container 13 (duplicate for container 14) was 6.6 after 24 hours. The pH values, after 24 hours, for the millipore and paper filtrates of the other batch of sterile culture medium used in experiment 9 were 7.2 and 7.0, respectively. The initial pH of month-old sterile culture medium and bacteria-free G. brevis cultures used in experiment 8 varied from 7.6 to 7.7. No pH values were ascertained initially in experiment 9, excepting the pH value of 7.8 for the week-old sterile culture medium used for eluting the millipore and filter paper residue.

The pH of culture medium either with or without G. brevis customarily drops after fish are introduced; this decrease probably results from increased bacterial growth and accumulation of fish waste products. Excepting two containers of culture medium (13 and 14) and a container of filter paper residues of a bacteria-free G. brevis culture eluted in sterile culture medium (4) of experiment 9, a minimal pH value of 7.0 was recorded after test periods as long as 24 to 30 hours in the two experiments (8 and 9) conducted with initially bacteria-free G. brevis cultures and sterile culture medium. Small mullet survived in culture medium with pH values as low as 7.0 and 7.1 in experiments 8 and 9.

We do not know why the pH of one particular flask of culture medium used in experiment 9 was so low. It is our surmise that the abnormally low pH resulted from failure to rinse the culture flask after hot nitric acid (7%) was used in the cleaning process. By actual test we found that failing to rinse the flask after the nitric acid treatment did signficantly lower the pH of 2.0 liters of sea-water-base medium. In this particular test the pH of the autoclaved medium stabilized at approximately 6.4.

A flask of culture medium, companion to one with the low pH (containers 13 and 14) of experiment 9, which was inoculated with G. brevis failed to support growth of this organism after incubating one month. Unfortunately, the medium in this flask was discarded without checking the pH. Since the two flasks of medium were prepared at the same time, we consider that G. brevis possibly failed to grow because the pH was unfavorable. We have experienced thus far only this one failure of bacteria-free G. brevis to grow in low-form culture flasks (3-liter). Growth of G. brevis has not occured in sea-water-base medium with an initial pH of less than 7.0. Furthermore, preliminary studies indicate that a pH below 7.4 is unsatisfactory for this organism.

The results of experiment 9a (Table 9) show that the culture medium in container 14 was no longer toxic to small mullet when retested about 24 hours after experiment 9 was begun. The pH of this medium was 6.4 about one hour after beginning experiment 9a.

All four test fish were alive after 24 hours; two of them were alive after 5 days. The pH of the medium in container 14 was 6.9 at this time.

The effects of millipore and paper filtration on the toxicity of bacteria-free G. brevis cultures were the same as observed when unialgal cultures were so treated. The more toxic portion of the culture passes through the millipore membrane whereas filter paper retains the more toxic fraction.

Bacteria-free G. brevis cultures proved just as toxic as the unialgal ones in simultaneous tests with comparable concentrations of this organism. For example, in experiment 8 (Table 6) the "death times" for M. cephalus in initially bacteria-free cultures varied from a minimum of 15 minutes to a maximum of 4 hours and 44 minutes. Seven of the 16 M. cephalus died in less time than was required (1 hour and 13 minutes) to kill the two M. cephalus subjected to a unialgal culture. The C. variegatus succumbed in the bacteria-free cultures in periods varying from a minimum of 3 hours and 18 minutes to a maximum of 32 hours; 13 of the 16 test fish died within 8 hours. Nine of the 16 C. variegatus in bacteria-free cultures died in less than the minimum time ($6\frac{1}{4}$ hours) required to kill the two C. variegatus in a unialgal culture. Further data for comparison of the effects of bacteria-free and unialgal cultures are available from experiment 9 (Table 8). Two large M. cephalus subjected to unialgal cultures died in 10 and 14 minutes. The four large mullet in the bacteria-free cultures died within 23 to 41 minutes. The "death times" for six small M. cephalus in the unialgal cultures varied from 15 minutes to $2\frac{1}{2}$ hours. The extremes of "death times" for the 12 small mullet in bacteria-free cultures were quite similar--25 minutes to 2 hours and 28 minutes.

In spite of the evidence that bacteria are not directly responsible for the toxic effects of G. brevis cultures, the possibility that bacteria play a significant role in the development of G. brevis "blooms" should not be overlooked. Such organisms may contribute appreciably to the nutrition of G. brevis. For example, some of the bacteria isolated from unialgal G. brevis cultures produce vitamin B_{12}-active substances (Starr et al., in press). Vitamin B_{12} apparently stimulates the growth of G. brevis in sea-water-base medium (Wilson and Collier, 1955).

Once fish kills are initiated by "blooms" of G. brevis, excessive bacterial growth resulting from the increased availability of organic matter may possibly cause the "blooms" to decline in isolated situations. Some of the ways in which bacteria could unfavorably affect G. brevis "blooms" include: producing substances toxic to this organism, competing for nutritive substances, and adversely altering the pH. We have frequently observed the failure of unialgal G. brevis to grow in tubes in which the medium became cloudy with bacterial growth.

Another role for which bacteria must be considered is that of a detoxicating agent. Shilo and Aschner (1953) found that bacteria decreased the toxicity of cultures of Prymnesium parvum, a marine and brackish water chrysomonad which is toxic to fish. Similarly, bacterial activity may influence the toxicity of G. brevis in the laboratory and in nature.

STUDIES WITH BACTERIA

Although the available evidence indicated that G. brevis causes the toxicity of unialgal cultures, direct proof was lacking prior to the development of bacteria-free cultures. In addition to the bacterial studies previouly considered, the possibility that bacteria may cause all or some of the toxic effects was investigated by testing pure cultures of some of the bacteria from unialgal G. brevis cultures. Furthermore, toxicity tests were conducted with pure cultures of an unidentified red-pigment-producing bacterium isolated from Florida waters and Flavobacterium piscicida Bein. Bein (1954) suggested that F. piscicida, a chromogenic bacterium, possibly was a cause of mass fish mortality associated with discolored water off the west coast of Florida.

Bacteria Isolated from Unialgal Gymnodinium brevis Cultures

Because of the preliminary nature of these studies and the crudeness of the quantitative bacterial estimates, only a summary will be presented. The bacteria used have not been identified; presently they are being characterized morphologically and physiologically by Dr. T. J. Starr. The test fish were Gulf killifish (Fundulus similis).

Two test fish per container (1-liter beaker) were subjected to about 500 ml of non-aerated test materials. Bacterial suspensions were prepared by adding 16.5 ml of a 24-hour culture to 500 ml of filtered Galveston Bay water. Control materials consisted of the same ratio of sterile culture medium and bay water as well as bay water alone. Crude estimates of bacterial concentrations were made by preparing a pour-plate of 0.02 to 0.03 ml of a sample collected shortly after the fish were added. A second sample was taken either after both fish died or after 23 hours if at least one fish survived this period. The colonies were too numerous to count in most of the plates prepared from the second samples. Therefore, not even rough estimates could be made for the bacterial concentrations.

The most abundant colony type isolated from unialgal G. brevis cultures on Spencer's peptone sea water agar and Bein's peptone agar is generally a convex white opaque colony produced by gram-negative rods. This colony type may represent several different species and physiological types of bacteria. Two separate isolates of the white opaque colony did not give evidence of being toxic to F. similis. The initial bacterial concentration was in the order of 0.5 to 1 million per ml.

A flat white transluscent colony with an iridescent sheen, also produced by gram-negative rods, is usually the second most abundant colony type isolated from unialgal G. brevis cultures. An initial concentration of approximately 1 million bacteria per ml of this colony type gave no evidence of toxicity to F. similis.

Chromogenic bacteria constitute only a small portion of the bacterial flora of unialgal cultures of G. brevis. However, yellow-pigment-producing bacteria become abundant in cultures treated with dihydrostreptomycin sulfate. They dominate in G. brevis cultures treated with 500 to 1000 µg of this antibiotic per ml, and often occur in nearly pure culture. This antibiotic may enhance the growth of the "yellow bacteria" by inhibiting competing bacteria. Dihydrostreptomycin sulfate (125 µg per ml) initially lowers the pH of culture medium by 0.5 to 0.8 of a pH unit. This change in the medium may be a factor favoring the increased growth of the pigment-producing bacteria.

Cultures of an isolate from a non-treated and a dihydrostreptomycin-treated unialgal culture, each with an initial count of about 1 million "yellow bacteria" per ml, had no toxic effects on F. similis. Plates prepared from samples taken 23 hours after the start of the experiment showed no yellow colonies. The counts of all bacteria were about the same in the initial and 23-hour samples.

A Chromogenic Bacterium Isolated from Water off the West Coast of Southern Florida

After Bein (1954) reported the toxic effects of Flavobacterium piscicida to fish, we made a cursory check for chromogenic bacteria in Florida off the Fort Myers-Naples area during November, 1954. G. brevis was present in the area at that time although the maximum concentrations were usually less than 1 million per liter. Small fish kills, mainly of mullet, were being reported sporadically at that time. During the sampling trips, however, we observed less than 10 dead fish. Surface samples from 15 stations in this area were plated on Spencer's sea water peptone agar. Four-plates, containing 1 ml of each sample, were prepared within 1 minute after collection to avoid possible changes in the bacterial flora. All except two of the samples from the 15 stations contained G. brevis, and the counts varied from 7,000 to 0.5 million per liter.

A white opaque colony was the most abundant type in the 15 samples; some samples showed a few lemon-yellow colonies. A total of two red colonies were observed in the 15 plates. One of these colonies was isolated from a sample taken 5 miles west of Wiggin's Pass on November 4, 1954. The G. brevis count for this sample was 8,000 per liter. The red-pigment-producing bacterium, which has not been identified, is a gram-negative motile rod.

A 24-hour pure culture of the "red bacterium" was tested for toxicity to F. similis as a part of the studies dealing with bacteria isolated from unialgal G. brevis cultures. Two fish were tested in each of the four containers of test material in which the bacterial count varied from approximately 0.5 to 1 million per ml. The bacterial culture gave a pink tint to the test materials. All eight fish died within 2 to 8 hours. After the last fish died in each of the four containers, samples were taken for bacterial counts. The red colonies in the plates prepared with 0.02 to 0.03 ml of these samples were so abundant that enumeration was impossible. We believe that the minimum concentrations were of the order of 1 to 2 million "red bacteria" per ml at the time the last fish died.

A 6-month-old unialgal G. brevis culture (replenished with fresh medium about three times weekly) containing 1.3 million organisms per liter killed the test fish less rapidly than cultures of the "red bacterium". In the G. brevis culture one fish died after $7\frac{1}{2}$ hours and the other one died after 10 to 19 hours. These "death times" appear relatively long when compared with the usual "death times" of fish subjected to other unialgal cultures. These results may mean either that F. similis is less sensitive to unialgal cultures than other fish tested thus far or that this culture was less toxic than the others.

Flavobacterium piscicida Bein

A chromogenic bacterium was isolated by Reuben Lasker (Bein, 1954) from a pooled water sample collected after the occurrence of a fish kill associated with discolored water in Whitewater Bay on the southwest tip of Florida. Bein (1954) found that 24-hour cultures of this bacterium, which he named Flavobacterium piscicida, killed several species of marine fish. He gave no quantitative values concerning the concentration of bacteria used other than that 500 ml of a 24-hour culture of this species grown in a 0.1% peptone solution in aged sea water were added to 4 gallons of continuously aerated sea water. After 24 hours all fish in the experimental aquaria died and the water exhibited a bright orange-yellow discoloration.

We attempted to estimate the minimum number of F. piscicida required to kill mullet (Mugil cephalus) since we desired to know what concentration of this bacterium might be required to kill fish under natural conditions. The Marine Laboratory of the University of Miami provided the stock from which our cultures were derived. Since Bein gave no indication of the amount of inoculum used to seed the medium to obtain the 24-hour test cultures, we could not duplicate his inoculation procedures.

In our experiment, 24-hour cultures were obtained by inoculating two loops of culture removed from a 24-hour slant culture (1% peptone

agar) into duplicate Erlenmeyer flasks containing approximately 150 ml of sterile 0.1% peptone solution in aged sea water. One flask was incubated at 30° C. and the other at 25° C. The culture incubated at 30° C. was deep orange after 24 hours and the one incubated at the lower temperature was yellow. The more intensely pigmented culture was used because it probably contained the greater concentration of bacteria. The ratio of bacterial culture volume to sea water volume varied from a maximum of 33.0 ml of 24-hour culture to 1.0 liter of sea water, which is about the same as used by Bein, to a minimum of one-hundredth of this ratio. Sea water and sterile 0.1% peptone solution were used as control materials. Each experimental container, 2-liter beaker, received 1.0 liter of sea water which was aerated continuously. Two mullet (3 to 4 in. long) were placed in each container. The test fish, which had been maintained in the laboratory for several days, were acclimated in the experimental containers for about 24 hours before beginning the study. The water which became cloudy in all containers by the end of the acclimation period was replaced with fresh sea water shortly before the test materials were added. Samples were collected for bacterial counts about 30 minutes after the experiment was begun. These samples were refrigerated $\frac{1}{2}$ to 2 hours before preparing the pour-plates.

All fish were alive when the experiment was discontinued 5 days later. A second set of bacterial-count samples were taken at this time. The samples were plated after being refrigerated $\frac{1}{2}$ to 3 hours. All bacterial plates were prepared with Bein's agar medium and they were counted after 6, 12, 15, and 21 days' incubation. The number of pigmented subsurface colonies as well as the pigment intensity of such colonies increased after prolonged incubation. After 21 days, however, some colonies began to lose pigment. Bacterial counts of the test materials used in this experiment are listed in table 10.

Discussion of Results of Studies with Bacteria

The results of the preliminary experiment with some of the bacteria isolated from unialgal G. brevis cultures suggest that cultures of the two dominant colony types, both non-pigmented, and a sparsely occurring pigmented form are not toxic to fish.

On the contrary, a "red bacterium" isolated from G. brevis-infested water off the west coast of Florida appears to be toxic to fish. Nevertheless, until this bacterium is found more abundantly or its association with fish kills is established, we shall not consider it of importance as a cause of fish mortality occurring off the west coast of Florida. Further toxicity studies with this organism have been discontinued until such time that evidence is obtained to implicate it as a fish-killing agent in nature.

A bacterium producing red pigment was isolated by Bein (1954) from Indian River on the east coast of Florida at the

Table 10.--Initial and final bacterial counts of test materials used in experiment with *Flavobacterium piscicida*[1/]

Container No.	Material in container other than 1.0 liter of sea water	Initial No. bacteria per ml (millions)	Initial No. *F. piscicida* per ml (millions)[2/]	No. bacteria per ml after 5 days (millions)	No. orange- & yellow-pigment-producing bacteria per ml after 5 days (millions)[3/]
1	none	0.80[4/]	none	1.6	0.050
2	33.0 ml sterile 0.1% peptone solution	0.46[5/]	none	1.7	0.060
3	33.0 ml 24-hour *F. piscicida* culture	5.0	1.9	3.6	0.010
4	16.5 ml 24-hour *F. piscicida* culture and 16.5 ml sterile 0.1% peptone solution	3.2	0.80	3.1	0.020
5	8.2 ml 24-hour *F. piscicida* culture and 24.8 ml sterile 0.1% peptone solution	1.2	0.42	2.5	0.040
6	Same as container 5	1.2	0.35	6.7	0.028
7	3.3 ml 24-hour *F. piscicida* culture and 29.7 ml sterile 0.1% peptone solution	0.40	0.22	1.8	0.050
8	0.3 ml 24-hour *F. piscicida* culture and 32.7 ml sterile 0.1% peptone solution	0.32	0.020	3.7	0.020

1/ The test fish (*Mugil cephalus*) showed no evidence of distress and none of them died during the 5-day test period.

2/ The number of *F. piscicida* based on the observed number of deep orange colonies.

3/ Since some of the chromogenic colonies varied from either yellow to orange or orange to yellow at various intervals during the 21 days of incubation, orange and yellow colonies were combined to indicate the maximum observed number of chromogens in this color range. There were no definitely orange colonies in samples from containers 3 and 8; about one-third of the pigmented colonies in the sample from container 6 and more than one-half of those in samples from all other containers were definitely orange.

4/ Approximately one-sixteenth of the bacteria produced yellow pigment.

5/ Approximately one-tenth of the bacteria produced yellow to light orange pigment.

time of an outbreak of discolored water during August 1951. Although the water was discolored when the isolation was made, no fish either dead or alive were observed. Bein found that this bacterium was nontoxic to several species of fish. Howell (1953) studied the same outbreak of discolored water. He reported that no great quantity of fish was killed; and that the discoloration was caused by a dinoflagellate, which he described as a new species, Gonyaulax monilata.

The attempt to determine the minimal lethal concentration of F. piscicida failed since none of the fish died during the experimental period. The reason for this lack of toxic effect is not known. Although we followed as nearly as possible the procedures used by Bein (1954), the experimental conditions employed were unfavorable for the bacterium, especially with regard to toxicity and pigment production. The bacterial counts given in table 10 indicate that high concentrations of F. piscicida (orange-pigment-producing bacteria) were present initially. However, the second counts, which were made from samples collected at the end of the 5-day experimental period, showed that either this chromogen or its chromogenic characteristic decreased greatly during the intervening period. A similar "disappearance" of chromogenic bacteria occurred during the toxicity study with "yellow bacteria" isolated from the unialgal G. brevis cultures. The experimental conditions may have affected the toxicogenic and chromogenic characteristics of this bacterium in several possible ways: (1) by killing the organism, (2) by inhibiting the growth of the organism, and (3) by altering the toxicogenic and chromogenic properties of the organism.

With only initial and terminal counts available the question as to when the "decline" of F. piscicida occurred--either early or gradually during the experimental period--cannot be answered. At no time during the 5-day period did the contents of any container show the bright orange-yellow discoloration observed by Bein after 24 hours. The contents of container 3, which received the maximum amount of F. piscicida culture, exhibited a tinge of orange after 24 hours. This slight discoloration gradually became less noticeable and completely disappeared by the fifth day.

The numbers of F. piscicida recorded in table 10 represent minimal counts, which may be considerably lower than the actual concentrations, because several of the white subsurface colonies produced the characteristic orange pigment after being transferred to agar slants (1% peptone). All deep orange colonies in plates prepared with samples which contained F. piscicida were considered to have been produced by this species. A few orange colonies appeared in plates prepared from some of the control samples,

particularly in those taken after 5 days. We made no allowance for orange colonies which possibly were not produced by F. piscicidia since the counts were considered low even with the inclusion of such colonies. An examination of the initial counts (Table 10) obtained for the control containers (1 and 2) and the two containers receiving the smallest volumes of bacterial cultures (7 and 8) suggests that counts of bacteria, exclusive of F. piscicida, varied in the order of 0.3 to 0.8 million per ml. From these values we presume that the actual concentration of F. piscicida was approximately twice as great as the values listed. For example, the initial number of F. piscicida in container 3 was probably about 4 million if one assumes that only about 1 million of the bacteria per ml were other than F. piscicida. The initial counts of this bacterium were in relatively good agreement with the various dilutions employed. For example, the material in container 3, which received the greatest volume of bacterial culture, yielded a count of about 2 million chromogens per ml whereas the material in container 8, which received only one-hundredth as much as container 3, gave a count of 20,000 chromogens per ml.

Since Bein gave only the ratio of F. piscicida culture and sea water employed, the concentrations of bacteria used in his studies cannot be directly compared with those we used. If the amount of water discoloration is proportional to the abundance of F. piscicida, we presume that the water in Bein's aquaria probably contained in excess of 4 million F. piscicida per ml at the end of the 24-hour experimental period. This presumption is based on the observation that the sea water in container 3 of our experiment was not appreciably discolored after the addition of 2 to 4 million F. piscicida per ml, whereas in Bein's studies the water was bright orange-yellow 24 hours after receiving the bacterial cultures.

GENERAL DISCUSSION

The well-established association of Gymnodinium brevis with the sporadic mass mortality of fish and other marine animals occurring in the Gulf of Mexico since 1947, in conjunction with the clear-cut laboratory demonstration that this dinoflagellate in pure culture is toxic to fish, leaves no reasonable doubt that this organism causes these mortalities. Considering this evidence, we propose the name "brevis red tide" for such mortalities instead of the nonspecific term "red tide" which is used commonly in popular and scientific writing. In our opinion there are ample characteristics to properly identify these mass mortalities occurring in the Gulf of Mexico as a distinct phenomenon. This phenomenon can be diagnosed by the presence of G. brevis in the waters in which fish and other marine animals are dying. An additional diagnostic characteristic is the

odorless human respiratory irritant often present in or near mortality areas where droplets of sea water become air-borne as a result of wind, wave action, etc. (Galtsoff, 1948; Gunter et al., 1948; Woodcock, 1948; Ingle, 1954; et al.).

Failure to find either one or both of the mentioned diagnostic characteristics in an isolated area of dead or dying fish would not necessarily eliminate G. brevis as the cause. There are at least four possible reasons for this statement. (1) The conditions or agents required to make droplets of sea water air-borne may be absent. (2) Dead fish may drift or be carried into an area either unsuitable for the survival of G. brevis or removed from the "bloom". (3) An isolated mass of water in which G. brevis is "blooming" may suddenly become unsuitable for this organism and yet not lose its toxicity to fish until sometime later. There is experimental evidence to support this suggestion since the removal of living G. brevis from cultures by millipore filtration or killing them with gentle heat did not inactivate the toxic substance. A specific diagnostic test for the toxic substance(s) produced by this organism would be helpful in diagnosing the cause of mortality in such cases. G. brevis is so delicate that under adverse conditions it may die within a matter of minutes leaving only fragmentary remains which are not readily identifiable. (4) The fish may contact the toxic substance in a "bloom" of G. brevis in one area and yet not succumb until moving into an area where the organism is not flourishing. This possibility is based on limited observations that fish exposed to G. brevis cultures for short periods and removed before showing distress, died after being placed in sea water. The "death times" after removal to sea water appear to decrease as the exposure time is increased.

Results of our studies with both heated and filtered G. brevis cultures, tests with other dinoflagellate cultures, and oxygen analyses of G. brevis cultures emphasize the existence of a toxic substance(s). The results of these studies support Galtsoff's (1948) conclusion that fish are not killed by clogging of the gill filaments by masses of G. brevis.

The available evidence makes untenable the view that fish suffocate as a result of mechanical occlusion of gill surfaces by the mere presence of large numbers of organisms. G. brevis cultures heated to 35 and 45° C. did not lose their toxicity although the organisms were disrupted. Likewise, the removal of this organism (both unialgal and bacteria-free) by millipore filtration did not detoxicate the cultures. Unialgal cultures of G. splendens and prorocentrum sp. were nontoxic in spite of the fact that the number of organisms compared with and even, in some cases, exceeded the concentrations in the toxic G. brevis cultures.

Filtrates of G. brevis cultures are toxic; however, the method of filtration determines whether the more toxic portion of the culture either passes through or is retained by the filter membrane. In our studies conducted with both unialgal and bacteria-free cultures, the filter paper residues eluted in either sea water or culture medium were more toxic to the fish than the filtrates. The results were reversed when a culture was passed through a millipore membrane under suction: the more toxic portion passed through the membrane. The reasons for the different effects of these two methods of filtration are not known. The filter materials differ in composition and size; the millipore membrane (diameter 47 mm) is made of cellulose esters whereas the filter paper (diameter 18.5 cm) consists of cellulose fibers. Retention of the toxic substance by filter paper may be due to greater adsorptive area and/or differences in physical and chemical properties. Another possibility is that filtration by gravity flow used with filter paper may result in the retention of considerably more intact organisms than in the case of millipore filtration under suction. Assuming that greater numbers of G. brevis were broken up by millipore filtration, more toxin might be released in such case. However, our preliminary studies showed no apparent increase in the toxicity of G. brevis cultures in which the organisms were cytolysed by gentle heating.

There are no indications that the fish kills caused by G. brevis result from the depletion of oxygen in sea water by great masses of this organism. Connell and Cross (1950) suggested that anaerobic conditions created by the high biochemical oxygen demand of an armored dinoflagellate, Gonyaulax, was the cause of mass mortality of fish associated with discolored water in Offatts Bayou (Galveston Bay) during the summer of 1949. Gunter et al. (1948) concluded that the 1946-1947 incidence of mass mortality of marine animals on the west coast of Florida was not associated with low oxygen. Oxygen deficiency can be excluded as a factor in the death of the fish in the G. brevis cultures which were aerated. In experiment 8 (Tables 6 and 7) the dissolved oxygen content of all aerated test materials exceeded 75% saturation and some were as high as 90%. With continuous gentle aeration one of the G. brevis cultures was about 90% saturated although the bacterial count was 24 million per ml at the time the dissolved oxygen was determined.

The results of an attempt to determine the effects of aeration on the toxicity of bacteria-free G. brevis cultures were contradictory. In experiment 8 the fish, especially M. cephalus, showed distress and died more rapidly in the aliquot which was aerated (dissolved oxygen--90% saturation) than in the nonaerated one (dissolved oxygen--45% saturation). The fish in another non-aerated aliquot (dissolved oxygen--55% saturation) in this experiment showed distress much sooner than those in the aerated culture

(dissolved oxygen--80% saturation). In experiment 9a (Table 9) M. cephalus died somewhat faster in the non-aerated aliquot of a G. brevis culture than in the aerated aliquot. It is apparent from these contradictory results that the influence of such factors as aeration cannot be evaluated until the standardization of the toxicity tests is more complete.

Several factors probably influence the degree of toxicity of G. brevis cultures. Aside from the concentration of G. brevis other factors such as the growth phase of culture, pH of culture during growth and during test period, temperature and salinity of test culture, size and number of test fish, volume and degree of aeration of test culture, and bacterial growth, are among those which must be considered in standarizing the toxicity tests. Shilo and Aschner (1953) found that a number of factors influenced the toxicity of Prymnesium parvum cultures. The toxicity was decreased by oxidizing agents, aeration, adsorbents including pond-bottom soils, bacteria growth, and low pH (below 7.5). They improved the standardization of their toxicity tests by using a buffer to control pH of the test culture and streptomycin to suppress bacterial growth. Furthermore, the test cultures were not aerated. McLaughlin (1956) working with the same organism reported that cultures grown in an alkaline medium were more toxic than those grown in an acid medium. P. parvum cultures (grown in alkaline media) rendered non-toxic by lowering the pH to 6.0 regain their toxicity when made alkaline (Shilo and Aschner, 1953; McLaughlin, 1956).

Some of the factors mentioned above may account for the variable "death times" obtained in duplicate containers of G. brevis cultures. Since on some occasions the pattern of response in one of the duplicate containers was different from that in the other, we consider that factors other than variations of the individual test fish were responsible. For example, in experiment 6 (Table 4) the fish died in 9 and 16 minutes in one container whereas death occurred after 1-2/3 and 2 hours exposure in another. Both containers (non-aerated) held similar amounts of the same unialgal culture. There are other anomalies which defy explanation at present.

One of these anomalies concerns the variation in the response of fish subjected to cultures supposedly grown under the same conditions, and treated in the same manner throughout the study. For example, in experiment 8 (Tables 6 and 7) container 9 received one of the duplicate pure cultures and container 10 received the other. The G. brevis counts of the material in each of the containers and such measured factors as pH, dissolved oxygen, and salinity were comparable. The similarity between the two containers is further emphasized by the "distress times" for the M. cephalus--10 and 12 minutes for container 9, 13 minutes for each of the two fish in container 10. In spite of all these similarities, the M. cephalus in container 9 died

in 15 and 25 minutes in contrast to 69 and 72 minutes in container 10. However, C. variegatus, a less sensitive fish than M. cephalus, showed more similarity of "death times": 3 hours and 51 minutes and 4 hours and 58 minutes in container 9, 3 hours and 44 minutes and 5 hours and 54 minutes in container 10.

Another anomaly in experiment 8 is the case in which one pure culture (container 12) with a G. brevis count of 3.4 million per liter was less toxic to both M. cephalus and C. variegatus than the duplicate culture (container 13) whose count was 2.3 million organisms per liter. The "distress times" (about $4\frac{1}{4}$ hours) and "death times" (about $4\frac{1}{2}$ hours) for M. cephalus in the more concentrated G. brevis culture (container 12) were approximately 2 hours greater than such times in the less concentrated duplicate culture (container 13). The "distress times" and "death times" for the C. variegatus in each culture were not as uniform as in case of the M. cephalus. Nevertheless, they show that the culture in container 13 was more toxic to C. variegatus than the one in container 12; the fish died after about 8 and 15 hours in the former and after about 27 and 32 hours in the latter container.

We realize that due to variations in the condition of the individual test fish some will survive longer than others when subjected to toxic agents. It is our opinion, however, that the over-all uniformity in the response of the test fish within each individual container, especially in experiment 8, is a strong indication of other subtle variables of which we have no knowledge.

Despite the evidence that our toxicity studies require more standardizing, the results of all experiments reported herein indicate that the sensitivity of fish to G. brevis cultures is variable. Our tests included six species of fish as follows: Membras vagrans, Mugil cephalus, Fundulus grandis, Mollienisia latipinna, Fundulus similis, and Cyprinodon variegatus. Possibly the most sensitive of these fish is M. vagrans; the only individual tested died in 4 minutes. M. cephalus, the species used in the greatest number of experiments (5), showed "death times" varying from a minimum of 8 minutes to a maximum of 4-3/4 hours. The great majority of them died within an hour. Small M. cephalus (1 to $1\frac{1}{4}$ in. long) are possibly slightly less sensitive to G. brevis cultures than large M. cephalus ($4\frac{1}{2}$ to $5\frac{1}{2}$ in. long). This possibility is suggested by results of experiment 9 (Table 8); the large M. cephalus in each container of unfiltered G. brevis culture, without exception, died before any of the three accompanying small M. cephalus. In some cases the first small mullet died within 3 to 5 minutes after the large mullet; in other cases the first small mullet died 20 to 70 minutes later. The F. grandis showed about the same degree of sensitivity as M. cephalus---minimum "death time" 9 minutes, maximum "death time"

2 hours and 10 minutes. The Mollienisia latipinna died in a minimum of 47 minutes and a maximum of 85 minutes. C. variegatus is probably the least sensitive of the six species tested. The minimum "death time" was about 2-1/3 hours, the maximum was 32 hours. The sensitivity of F. similis is possibly comparable to that of C. variegatus. Two F. similis died in 7½ and 10 to 19 hours.

A chromogenic bacterium, Flavobacterium piscicida, has been suggested as a possible cause of the mass fish kills and associated sea water discolorations occurring along the west coast of Florida (Bein, 1954). Bein found that this bacterium was toxic to several species of fish although he did not indicate the bacterial concentrations employed. In our tests Mugil cephalus were not affected by initial concentrations of 2 million or more F. piscicida per ml. Contrary to Bein's experience, F. piscicida apparently did not grow in our experiment and possibly lived only a short time after being added to the test medium (sea water). We could scarcely detect this bacterium at the end of the 5-day test period. A "red bacterium" which we isolated from G. brevis-infested water off the west coast of Florida appears to be toxic to fish. Concentrations of the order of 0.5 to 2 million bacteria per ml were toxic to Fundulus similis. The "red bacterium" has not been encountered in an abundance to implicate it as a fish-killing agent. Thus far, neither the association of chromogenic bacteria with extensive fish kills nor the natural existence of toxic concentrations of such bacteria has been established. In the case of Gymnodinium brevis both of these conditions have been well established.

SUMMARY AND CONCLUSIONS

1. Since 1947 "blooms" of the dinoflagellate, Gymnodinium brevis, have been associated with sporadic mass mortalities of marine animals and discolored water in the Gulf of Mexico. Extensive laboratory studies conducted with unialgal and bacteria-free cultures as well as related bacterial studies offer overwhelming evidence that "blooms" of this organism are the direct cause of the associated mortalities.

2. Bacteria-free cultures of G. brevis with concentrations varying from 2.3 to 4.8 million organisms or liter were toxic to three species of test fish. Five species of fish were killed when subjected to unialgal G. brevis cultures containing 0.6 to 2.1 million organisms per liter. The numbers of G.brevis in areas of natural fish kills often greatly exceed these toxic laboratory concentrations.

3. Bacteria apparently do not produce or directly contribute to the production of the toxic substance present in G. brevis cultures. Bacteria-free cultures were just as toxic to fish as the unialgal ones.

4. The toxicity of G. brevis does not depend on the presence of living organisms. Removing the organisms from cultures by millipore filtration or killing them with gentle heat did not appear to alter the toxicity. The high dissolved oxygen content of aerated G. brevis cultures eliminates oxygen deficiency as the cause of toxicity.

5. The toxic substance produced by G. brevis readily passes through a millipore membrane, but for the most part is retained by filter paper.

6. Studies with bacteria-free and inialgal cultures indicate that the six species of test fish are differentially sensitive to G. brevis cultures. The test fish, listed in order of decreasing sensitivity, were: Membras vagrans, Mugil cephalus, Fundulus grandis, Mollienisia latipinna, Fundulus similis, and Cyprinodon variegatus.

7. Some chromogenic bacteria isolated from the Gulf of Mexico or adjoining bays are toxic to fish in laboratory tests. However, an association of such bacteria with mass fish kills in the Gulf of Mexico has not been established. Flavobacterium piscicida, previously found to be toxic to several species of fish in undetermined concentrations, was not toxic to Mugil cephalus at an initial concentration of about 2 million per ml. A "red bacterium" isolated from G. brevis-infested waters appears to be toxic to fish at concentrations in the order of 0.5 to 2 million per ml. This bacterium was uncommon during our survey since only two colonies were obtained from 15 samples of 1.0 ml each.

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